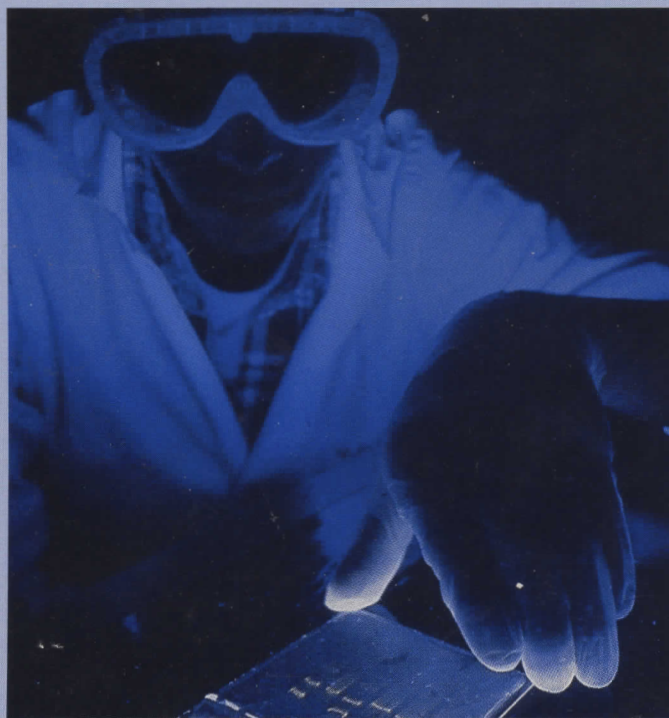


Introduction to Experimental Molecular Biology

Holly Ahern



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Introduction to Experimental Molecular Biology

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State University of New York, Albany



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Preface

Three years ago I was given the challenge to create a new laboratory course in molecular biology. An immediate search began for a suitable laboratory manual. However, the lab manuals I encountered were much too advanced for use in an introductory molecular biology laboratory course. I needed a text that began with the basics, since this would be the first exposure to experimental molecular biology for many of the students taking the course. I also wanted a manual that revolved around a central theme because I learned from experience that giving students more responsibility, in terms of growing and maintaining cells and then preparing DNA from those carefully kept cells, made them more actively involved and interested in the experiments than if they were simply mere observers.

In writing this text, I tried to create a manual that could cover the important introductory subjects and, over the course of a semester, build upon these introductory skills and have students solve more complex and scientific problems. My goal was to have the students taking this course, mostly junior and senior level biology majors, leave with a sense of what experimental science was really all about. Many of the manuals currently on the market take a "real science" approach to molecular biology experiments. The exercises begin on one day, and then continue for several days, which is what really happens in most research. However, it is sometimes impossible for students at colleges and universities to fit one or two hours of lab time into their busy schedules every day. Laboratory sessions here at the University are scheduled to meet once a week for four hours, and the exercises in this manual conform to that schedule as much as possible without losing the feel for what really goes on in research laboratories. On some occasions, students will need to spend additional time in the lab, but only for a short time—to subculture for the next day or to pick up results. The laboratory exercises described in this manual are flexible enough so that they could be split into two lab periods meeting for two or three hours each week. Notations have been included in the procedures to point out places at which an experiment could be put on hold. In most cases, the experiments can be molded at the discretion of the instructor to fit each individual laboratory class's schedule.

The experiments in this manual have been designed to revolve around a single gene in order to maintain continuity and to allow students to become more actively involved in the experimental process. The experiments are also useful for scientists who are just beginning to move into the molecular biology field and want to learn the basics before tackling crucial experiments; in that respect, the manual can be used as a basic reference guide. The beginning chapters introduce the basic skills of molecular biology, microbiological techniques, restriction digestions, and agarose gel electrophoresis. Once the basics are mastered, the text moves into a detailed analysis of the gene, using more advanced methods in molecular biology and genetics.

The goal of this project was to create a meaningful and creative laboratory experience for students who are first opening their eyes to the exciting world of molecular biology. Perhaps these students will be inspired to continue in the field after they graduate. Even if they do not, it will provide them with the skills necessary to work or study in many other related areas, such as medicine, environmental studies, or biotechnology. Molecular biology has become firmly entrenched as the science of the future, and it is time to awaken our students to this fact.

A special thanks goes to Oscar H. Will, Augustana College and Ron W. Leavitt, Brigham Young University for reviewing this text.

Notes to Instructors

When I first began teaching laboratory courses many years ago, I quickly learned that the way to insure the success of lab exercises was to keep the experiments as simple as possible for both the students and the instructor. Fortunately, many biological supply companies have the same opinion. Many companies sell the specialized reagents and equipment required in a molecular biology lab at a cost comparable to homemade. Several biotechnology companies also market kits for molecular biology procedures that represent a substantial time savings for the user. Gradually, the courses I taught (as this manual does) evolved to the point where the kits and premade reagents were often employed for use in preparation of supplies prior to the labs, and sometimes during the experiments by the students themselves. The reasons to support this are many. When you use a kit or reagent from a biological supply company, the expertise of their technical support staff is only a phone call away. Another reason is the enormous savings in preparation time required for each lab and during the actual experiment as well. For example, in our laboratory the Southern blot went from an overnight experiment everyone dreaded to a half-hour procedure using the VacuBlot apparatus. This equipment alone allowed me to fit a Southern blot experiment into a single lab period.

Students learning molecular biology for the first time sometimes have difficulty learning to deal with the microvolumes used when working with DNA. Because of this, it is strongly recommended that the instructor make or buy quantities of plasmid and genomic and lambda DNA ahead of time, just in case student yields are low. This task can be greatly simplified through the use of two kits. The CirclePrep kit from BIO 101 is a rapid and inexpensive method for the extraction of plasmid DNA from bacterial cells. Large (microgram) quantities of plasmid DNA that are free from RNA or chromosomal DNA contamination can be prepared in less than two hours. To prepare genomic DNAs, the Extractor kit from Molecular Biosystems, Inc. described in chapter 10 can be used with guaranteed results.

Laboratory conditions vary greatly from one place to another, especially where students are concerned. To fully insure that the exercises described in the lab manual are a success, it is recommended that the instructor perform the experiments well in advance of the scheduled lab period. In this way, it will be possible to make adjustments in the procedures if necessary, to better fit the laboratory schedule or laboratory conditions.

Figure 1. Standard curve generated from the digestion of lambda DNA with the restriction enzyme Hind III.

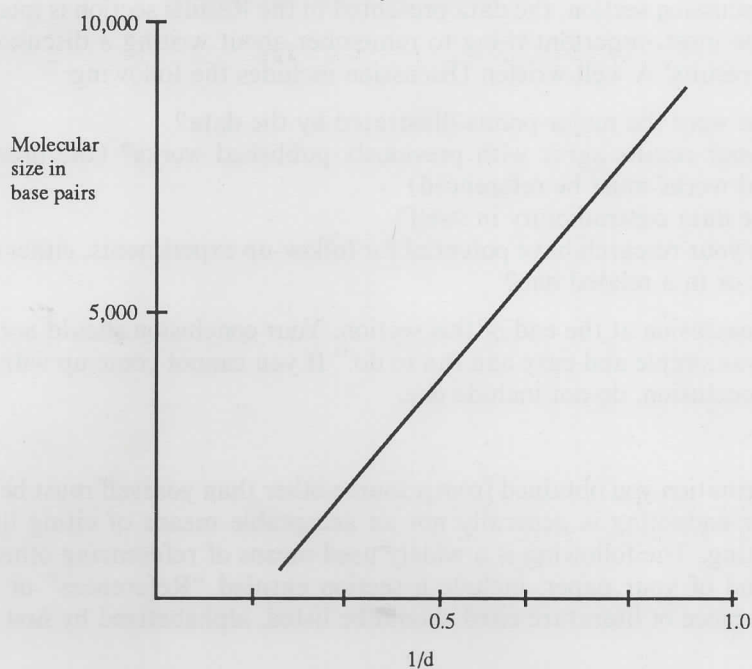


Table 1. Restriction fragment sizes determined from the digestion of the recombinant plasmid pMB1 with the restriction enzyme Pvu II.

Band	Distance from Well (cm)	1/d	Molecular Size (in base pairs)
1	3.2	0.31	4,500
2	5.0	0.20	2,000
3	5.6	0.18	1,750

Discussion

In the Discussion section, the data presented in the Results section is interpreted and discussed. The most important thing to remember about writing a discussion is not to reiterate the results! A well-written Discussion includes the following:

1. What were the major points illustrated by the data?
2. Do your results agree with previously published works? (the previously published works must be referenced)
3. Is the data contradictory in itself?
4. Does your research have potential for follow-up experiments, either on the same topic or in a related one?

State a conclusion at the end of this section. Your conclusion should *not* be "... the experiment was simple and easy and fun to do." If you cannot come up with a pertinent and logical conclusion, do not include one.

References

Any information you obtained from a source other than yourself must be referenced. Footnoting or endnoting is generally not an acceptable means of citing literature for scientific writing. The following is a widely used means of referencing other works.

At the end of your paper, include a section entitled "References" or "Literature Cited." Each piece of literature cited should be listed, alphabetized by first author, and numbered.

1. Baxter, W., and Bowen, W. 1980. Experimental Cell Biology, MacMillan Pub. Co. Inc., NY.
2. Dorin, J. R. 1987. "A Clue to the Basic Defect in Cystic Fibrosis from Cloning the CF Antigen Gene." Nature 326:614-617.
3. Gold, L., and Stormo, G. 1987. Escherichia coli and Salmonella typhimurium: Cellular and Molecular Biology, Vol. 2, American Society for Microbiology, Washington.
4. Wolfe, S. A., and Smith, J. M. 1988. "Nucleotide Sequence and Analysis of the purA Gene Encoding Adenylsuccinate Synthetase of E. coli K-12." J. Biol. Chem. 263:19147-19153.

In the text of your paper, cite your references as follows:

This result agrees with the data obtained by Baxter and Bowen (1) . . .

The purA gene of E. coli encodes the protein adenylsuccinate synthetase . . . (4) . . .

Dorin and his coworkers have demonstrated that . . . (3) . . .

Remember, when in doubt cite it! It is better to reference too much than not enough!

Chapter 1

Introduction to Molecular Biology

Speculation regarding the nature of heredity has occurred throughout history. Even very primitive cultures knew that certain traits, such as hair or eye color, could be passed on within a family. The real search for the actual genetic material began in the early 1860s, largely due to the efforts of an Austrian monk, Gregor Mendel. Mendel's research centered around the inheritance of specific traits in pea plants. His observations led him to postulate that heredity involved the physical transmission of traits from parent to offspring. His work went mostly unnoticed until the turn of the century, when it was determined that the heredity of traits occurred via the gametes (the egg and the sperm). When sperm fertilized an egg, the resulting cell contained chromosomes from each parent. Thus, as Mendel predicated, the heredity of traits was linked to cellular structures, the *chromosomes*.

By the late 1930s, research on the genetics of the fruit fly *Drosophila* yielded evidence indicating that each chromosome contained a large number of smaller units called *genes*. Shortly thereafter, in the early 1940's, George Beadle and Edward Tatum showed that there was a connection between chromosomal mutations and protein deficiencies in the bread mold *Neurospora*. When Linus Pauling linked the disease sickle cell anemia to a mutation that affected production of hemoglobin in red blood cells, the one gene, one protein theory, which stated that each gene on a chromosome made a single protein product, was born.

The physical nature of chromosomes remained a mystery, however. As long as the molecular basis of chromosomes was unknown, there was no way to constructively relate genes and proteins. In the 1940's chromosomes were purified away from other cellular components and studied, in an effort to determine their structure and composition. It was determined that eukaryotic chromosomes were made of deoxyribose nucleic acid (DNA), and small positively charged proteins called *histones*. Eventually, thanks to the efforts of Fredrick Griffiths in 1928, and later Oswald Avery and his colleagues Maclyn McCarty and Colin MacLeod, it became apparent that the DNA, and not the protein, was the component responsible for the heredity of genetic information. In 1953, James Watson and Francis Crick, using previously determined biochemical and physical data on DNA, proposed the three-dimensional structure of DNA. Their model showed DNA as composed of two antiparallel chains of nucleotides held together by the bonds formed between "base-pairs" of *pyrimidine* (*thymine* and *cytosine*) and *purine* (*adenine* and *guanine*) bases, as shown in figure 1.1.

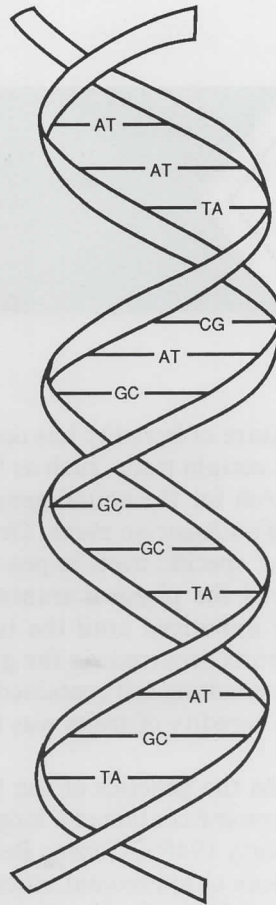


Figure 1.1. The DNA double helix, as proposed by Watson and Crick.

The discovery of the *DNA double helix* touched off the explosion that led to the birth of molecular genetics as a science. It soon became apparent that the double helical antiparallel strands of DNA could be separated, and each strand replicated to yield daughter strands that were identical copies of the parent. It also became obvious that DNA directed the process of protein synthesis, and that each gene on a chromosome was the template for the production of one cellular polypeptide. This led Crick to propose what he called the “Central Dogma” of biology; that the genetic material for each cell was found in its DNA, which could be replicated to yield identical daughter DNA, or transcribed into a messenger nucleic acid, messenger RNA (mRNA). Messenger RNA, then, was the intermediate between DNA and protein, with protein synthesis directed by the information found in the nucleotide sequence of the RNA molecule.

First Position	Second Position				Third Position
	U	C	A	G	
U	PHE	SER	TYR	CYS	U
	PHE	SER	TYR	CYS	C
	LEU	SER	STOP	STOP	A
	LEU	SER	STOP	TRP	G
C	LEU	PRO	HIS	ARG	U
	LEU	PRO	HIS	ARG	C
	LEU	PRO	GLN	ARG	A
	LEU	PRO	GLN	ARG	G
A	ILE	THR	ASN	SER	U
	ILE	THR	ASN	SER	C
	ILE	THR	LYS	ARG	A
	START	THR	LYS	ARG	G
G	VAL	ALA	ASP	GLY	U
	VAL	ALA	ASP	GLY	C
	VAL	ALA	GLU	GLY	A
	VAL	ALA	GLU	GLY	G

Figure 1.2. The genetic code is found in mRNA. Each codon, which consists of a set of three nucleotides, is translated by ribosomes that read the codons and add the correct amino acid onto the growing polypeptide chain.

Conversion of the information contained in a strand of DNA into a biologically active protein is a complex process. First, one of the strands of a DNA molecule is enzymatically copied, or *transcribed*, into an intermediate single-stranded polynucleotide, *ribonucleic acid (RNA)*. This molecule is structurally similar to DNA, except that RNA contains the base uracil in place of thymine. In eukaryotes, the RNA is then transported from the nucleus, where the chromosomes are found, to the cytoplasm. In the cytoplasm, ribosomes attach to the mRNA and *translate* the message into protein. In prokaryotes, transcription and translation are coupled; as soon as a strand of mRNA is synthesized, ribosomes attach and protein synthesis begins. Ribosomes are made up of proteins and several alternate forms of RNA (rRNA). The ribosomes bind to one end of a molecule of mRNA at a specific three-base sequence (usually AUG) and translate the sequence of nucleotides into amino acids. The ribosomes do not read every base in the mRNA; instead, they use three base sequences, or *codons*. Each codon specifies one amino acid, but each amino acid can be specified by more than one codon. Thus, the genetic code is termed *degenerate* (see figure 1.2). The growing chain of amino acids forms the protein product.

Regulation

The genetic code is maintained in nature and has been found to be highly conserved, even among widely divergent species. The differences among organisms lie in the mechanisms of regulation, or the way that genes encoded in DNA are expressed. Cells have evolved a means to save energy by regulating the expression of their genes. Generally,

if a protein is not needed by the cell at any given time, the gene encoding that protein will not be transcribed. Transcription of a gene into mRNA will not occur unless the appropriate biochemical signal is sent to the transcription machinery. In some cases, the signal takes the form of a *repressor molecule*, which binds to DNA and inhibits the action of RNA polymerase, the enzyme that catalyzes the transcription reaction. When a repressor is present, the gene is not expressed. Removal of the repressor results in expression of the gene and synthesis of its encoded protein. For other genes, the signal is an *activator protein*, which must first bind DNA before RNA polymerase can bind it. In this case, a gene is expressed only when the activator protein is present. Regardless of the mechanism or the organism in which it occurs, gene expression is an important cellular function, and strict regulation is critical to survival.

Much of our knowledge about the nature of genetic systems and their regulation came as a result of work with prokaryotic organisms, especially bacteria. Many scientists study genes and their regulation in the bacterium *Escherichia coli*. *E. coli* is a non-pathogenic bacteria found primarily in the intestines of warm blooded animals. There are many reasons for using *E. coli* in genetic studies. First, *E. coli* can be easily cultivated in the laboratory, using media containing only a carbon source and a few inorganic compounds. This simple type of media is called minimal media. Mutant *E. coli* strains are easily detected by their inability to grow on minimal media. When the specific nutrient required by the mutant is added to the media, the bacteria will be able to grow. This provides scientists with a simple means for genetic selection of auxotrophic strains. Additionally, *E. coli* is a haploid organism, whose simple chromosome has been both genetically and physically mapped. *E. coli* has a short generation time (20 minutes) and, therefore, the results of genetic experiments are available after a few short hours, as opposed to years. As an added feature, *E. coli* can carry in its cytoplasm small autonomously replicating, extrachromosomal pieces of DNA called *plasmids*. Plasmids encode genes that are basically nonessential to the bacterium's existence, but provide a selective advantage, such as resistance to antibiotics. Plasmids are transmissible between bacteria of the same species, and can be used to introduce new genetic material into a bacterial cell.

Genetic Engineering and Molecular Biology

Molecular biology is the study of biological molecules: DNA, RNA, and protein. One of the most important recent discoveries in this field was the development of *genetic engineering*, or *recombinant DNA technology*. In the late 1960s, enzymes were discovered in *E. coli* that recognized specific DNA sequences and cleaved the DNA at highly specific sites within the recognized sequence. These enzymes were called *restriction endonucleases* because their cellular function was to restrict the entry of foreign DNA into the cell by cutting it up. They were shown in cell-free extracts to work only on foreign DNA. Bacterial cells methylated their own DNA, thus protecting it from cleavage by restriction enzymes. Scientists were at first very excited about this discovery because it provided them with a means of rearranging genetic material artificially. However, the first restriction enzymes did not live up to the expectations of the researchers because cleavage sites were random and distant from the unmethylated restriction sites.

Table 1.1. Source and specificity of some restriction endonucleases. Some enzymes leave staggered cuts in double-stranded DNA (BamH I) while others leave blunt ends (Sma I). A restriction enzyme recognition sequence generally contains between four and eight nucleotides.

Enzyme	Recognition Sequence	Organism
BamH I	G [↓] GATCC CCTAG [↑] G	Bacillus amyloliquefaciens
Bgl II	A [↓] GATCT TCTAG [↑] A	Bacillus globigii
Cla I	AT [↓] CGAT TAGC [↑] TA	Caryophanon latum
EcoR I	G [↓] AATTC CTTAA [↑] G	Escherichia coli RY13
Hind III	A [↓] AGCTT TTCGA [↑] A	Haemophilus influenzae Rd
Not I	GC [↓] GGCCGC CGCCGG [↑] CG	Nocardia otitidis-carviarum
Sau3A 1	[↓] GATC CATG [↑]	Staphylococcus aureus 3A
Sma I	CCC [↓] GGG GGG [↑] CCC	Serratia marcescens Sb
Xho I	C [↓] TCGAG GAGCT [↑] C	Xanthomonas holcicola

Then in 1970, restriction enzymes were found that did recognize and make double-stranded cuts at specific unmethylated sequences in DNA. These enzymes became known as *type II restriction endonucleases*. The discovery of the type II enzymes opened the door to macromolecular research. In the years between 1970 and the present, over 250 restriction enzymes from a number of different bacterial sources have been found, giving rise to a pool of enzymes that allow a researcher to cleave DNA at virtually any site within a genome. Table 1.1 lists a few of these enzymes and their bacterial source.

One of the first applications of restriction enzyme technology was to cleave the DNA of various viruses to determine the location of different restriction sites on the viral genome. This technique was quickly adapted to other types of DNA, such as plasmids. It was determined that some restriction enzymes, such as EcoRI, leave short, single-stranded tails, called *sticky*, or *cohesive, ends*, that arise from the staggered cuts made in the two strands of DNA. It became intuitively obvious that the single-stranded overhangs could base-pair with complimentary overhangs from other pieces of similarly cleaved DNA. Thus, any two fragments of DNA, regardless of their origin, can be combined into a single DNA molecule by way of their sticky ends, creating a recombinant DNA molecule.

This technology paved the way for gene cloning. A piece of DNA containing a gene of interest can be inserted into a plasmid carrier, or *vector*, by cutting the plasmid and target DNA with the same restriction enzyme, generating fragments with sticky ends. These fragments are reannealed in the presence of another bacterial enzyme, DNA ligase, creating a recombinant DNA molecule. This recombinant plasmid can then be inserted, or transformed, across the cell membrane into a suitable bacterial cell host. When the bacterial cell replicates, the recombinant plasmid will be replicated as well. Many plasmids also replicate autonomously to maintain a high copy number, or more than one plasmid, per cell. Bacterial cells containing a recombinant plasmid can be stored, or the gene on the plasmid can be expressed *in vitro* to obtain large quantities of gene product. Once a gene is cloned, any number of different technologies can be applied to mutate, manipulate, or sequence the gene. The ultimate goal of these types of experiments is usually to determine the exact function of the gene.

It is important to note that recombinant DNA technology is a researchers' tool to be used at the discretion of the molecular biologist, and is not a science in itself. Recombinant DNA techniques must be appropriately applied to a scientific problem before it can be solved, and it is the correct application of a variety of techniques that has led to the very exciting recent advances in molecular biology. The goal of this manual is to provide the knowledge and techniques needed to successfully study a genetic system and a particular gene, the *purA* gene of *E. coli*. These techniques can be easily applied to any other system with equivalent results.

The *purA* Gene of *E. coli*

Purines (adenine and guanine) and pyrimidines (cytosine and thymine) are the bases found in DNA and RNA. These bases are not normally found as free bases in the intracellular state. Instead, they are synthesized as *nucleotides*, consisting of a phosphate group, a sugar, and a purine or pyrimidine base linked to the sugar via a glycosylic bond. A molecule of DNA or RNA consists of a chain of nucleotides hooked together by phosphodiester bonds between the phosphate group of one nucleotide and the sugar, either ribose (in RNA) or 2-deoxyribose (in DNA), of the adjacent nucleotide. Thus, the production of stable RNA or DNA molecules in a cell requires the synthesis of purine and pyrimidine nucleotides. In addition, the nucleotides *ATP* and *GTP* are the energy sources required to drive many different biosynthetic processes.

Nucleotide triphosphates such as *ATP* are synthesized from monophosphate precursors after a series of phosphorylation reactions. These sequential phosphorylations are catalyzed by a cellular enzyme, *kinase*. The precursor of the purine ribonucleotides *ATP* and *GTP* is *IMP*, *inosine monophosphate*. *IMP* is the first purine nucleotide formed in the pathway for synthesis of *ATP* and *GTP*. (see figure 1.3)

In this pathway, *AMP* is first synthesized from *IMP* in a two-step reaction. First, a condensation reaction occurs between the amino acid aspartate and the sixth position

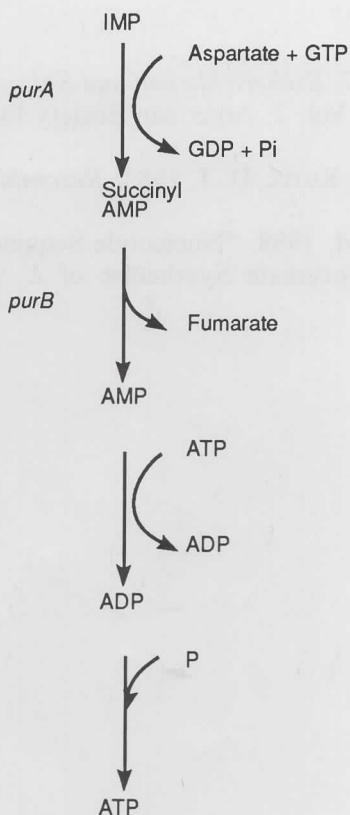


Figure 1.3. The *purA* gene product, adenylysuccinate synthetase, catalyzes the first step of ATP biosynthesis; the synthesis of adenylysuccinate (succinyl AMP) from IMP.

carbon of IMP, to yield succinyl AMP, which is quickly converted to AMP. The monophosphate nucleotides are then successively phosphorylated to give rise to the triphosphate form, ATP. The *purA* gene product, adenylysuccinate synthetase, catalyzes the first step in the pathway. Therefore, *E. coli* cells mutant at the *purA* locus are adenine auxotrophs, requiring adenine for growth.

Because of its role in purine ribonucleotide synthesis, the *purA* gene has recently become the focus of research. The *purA* locus has been mapped and cloned, and its base sequence has been determined. These analyses have shown that the *purA* gene maps to 95 minutes on the *E. coli* genetic map, and is apparently unlinked to the other purine biosynthesis genes in the pathway. Adenylysuccinate synthetase, the *purA* gene product, is a dimer, consisting of two subunits of 48,000 Da each. Regulation of this gene results from feedback inhibition and by the availability of a suitable substrate. The *purA* gene, because of its importance and relative ease of manipulation, will be the model system used throughout this manual.

Suggested Readings

- Gold, L., and Stormo, G. 1987. *Escherichia coli* and *Salmonella typhimurium: Cellular and Molecular Biology*, Vol. 2. American Society for Microbiology, Washington, DC.
- Watson, J. D.; Tooze, J.; and Kurtz, D. T. 1983. *Recombinant DNA: A Short Course*. Scientific American, Inc.
- Wolfe, S. A., and Smith, J. M. 1988. "Nucleotide Sequence and Analysis of the *purA* Gene Encoding Adenylylsuccinate Synthetase of *E. coli* K-12." *J. Biol. Chem.* 236: 19147-19153.

NOTES AND CALCULATIONS

Chapter 2

Basic Microbiology and Aseptic Technique

Introduction

Scientists have long recognized the importance of bacteria to the field of biology. Bacteria were first studied on the basis of their medical importance. It was quickly realized, however, that bacteria were extremely useful in genetic studies for many reasons. First, bacteria are haploid. If a mutation arose in a single bacterial gene, it would be easily detected phenotypically. Bacteria also have a rapid rate of growth. For example, an *Escherichia coli* cell divides to form two daughter cells on the average of every twenty minutes. Therefore, the inheritance of genomic traits could be followed through many generations in a relatively short period. Bacteria are also subject to genetic variation by mutation, which is useful in the study of expression or regulation of a particular gene. New genetic material can be introduced into bacterial cells by *conjugation*, or mating; by *transduction*, the infection of a bacterium by a bacterial virus (bacteriophage); or by *transformation*, which is the introduction of free DNA into the cell across the cell membrane. Bacteria are also used in molecular biology studies because they can be grown in large quantities, and specific genes or gene products can be isolated from the cultured cells.

The bacterium most commonly used in molecular biology is *Escherichia coli*. *E. coli* is a nonpathogenic enteric bacterium that can be easily cultivated in the laboratory. Extensive genetic studies on the bacterium have generated a complete map of its single circular chromosome. Many different auxotrophic *E. coli* strains are commercially available for use in research.

In order to cultivate *E. coli* or other bacteria successfully in the laboratory, it is necessary to reproduce optimal growth conditions artificially. Bacterial growth requirements include nutrients such as carbon or nitrogen sources, vitamins, and water, and physical factors such as the proper temperature, pH, and gaseous environment. The nutritional needs of bacteria can be supplied through microbiological media, which usually contain protein extracts such as peptone, inorganic salts, and in some cases, a complex carbohydrate such as dextrose or lactose. The two most common forms of media are *broth media*, in which bacterial cells grow in suspension, and *plate media*, which contains a solidifying agent such as agar in addition to the nutrients.

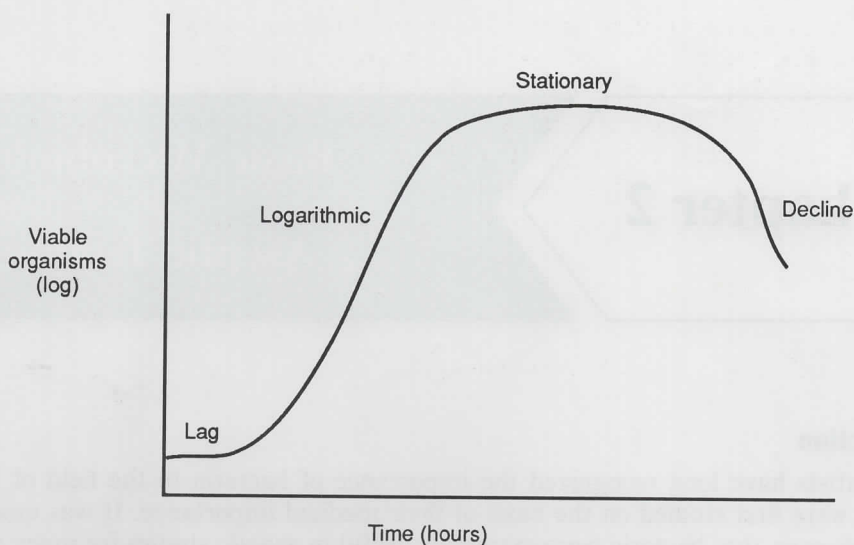


Figure 2.1. The bacterial growth cycle includes four stages; the lag stage, the logarithmic stage, the stationary stage, and the stage of decline.

If a vessel filled with medium is inoculated with bacteria and incubated under optimal growth conditions, the bacteria will replicate and the culture population will grow in number. The dynamics of bacterial growth can be plotted using a population growth curve, which plots the increase in cell number versus time as shown in figure 2.1. From this curve, it is seen that growth of a population of bacteria can be delineated into four discrete stages. The first stage, or *lag stage*, represents the adjustment of bacteria to the new media. The second, or *logarithmic stage*, is characterized by rapid cell division and an exponential increase in the size of the population. Following the log phase is the *stationary phase*, in which the number of dividing cells is roughly equal to the number of dying cells. Finally, in the *stage of decline*, depletion of nutrients in the system and the build-up of metabolic wastes results in the uniform death of the bacteria in the culture.

The nature of bacterial cell growth predicts that a culture of bacterial cells will quickly become nonviable if they are not subcultured. Subculturing involves a transfer of cells from an old culture into a new media. This transfer must be done aseptically (sterilely) because other microorganisms from the environment can easily contaminate the culture. In many cases, the subculturing procedure involves dilution of the original culture either before or during transfer, to insure that single, isolated colonies are formed. Each colony that forms on solid media represents descendents of a single bacterium all growing in one small lump on the surface of the medium. Therefore, the bacteria contained in a colony represent genetic clones of the original cell.

In molecular biology, two techniques are commonly used to isolate pure cultures. The *streak-plate technique* is essentially a dilution performed by streaking a loopful of bacterial culture over the surface of an agar plate. The second method involves physically diluting a dense culture in medium or saline, and then spreading a small volume of the

dilute culture on an agar plate using an L-shaped glass rod while the plate is spun on a small turntable. The first method is most often used to isolate single colonies, while the second is most often used to quantitate the number of viable bacteria in a culture.

The objective of this experiment is to familiarize students with the techniques most commonly used in the aseptic transfer of bacteria during subculturing. Procedures requiring the use of aseptic technique involve manipulations that may seem awkward at first. Practice is necessary to master the art of sterile technique.

Materials

Media

TY broth, TY agar plates, TY agar slants

Reagents

95% ethanol, sterile water

Supplies

Inoculating loop; Bunsen burner; turntable; glass spreader; sterile dilution tubes; sterile culture tubes; 1-, 5-, and 10-ml serologic pipettes; test tube rack

Cultures

TY broth culture of *E. coli* C600 grown overnight at 37° C

Procedure

A. Subculturing to Liquid Medium

1. Aseptically dispense 2 ml of TY broth into a sterile culture tube as follows:
 - a. Remove the cap from a bottle containing TY broth. Do not put the cap down.
 - b. Flame the mouth of the bottle. Then, holding the bottle at an angle, insert a sterile 5-ml serological pipette so that the tip just barely breaks the surface of the media. Draw up 2 ml.
 - c. Remove the pipette and recap the bottle.
 - d. Remove the cap from a sterile culture tube by grasping it in the pinky of the hand holding the pipette (see figure 2.2). Dispense the TY broth into the tube.
 - e. Flame the neck of the culture tube and replace the cap.
2. Sterilize an inoculating loop by placing it in the hottest part of the flame of a Bunsen burner. Once the loop has been flamed, do not put it down or touch it to any other surface. Allow the loop to cool for ten to fifteen seconds.
3. Remove the cap from the tube containing the *E. coli* overnight culture by grasping it with the pinky finger of the hand holding the loop.
4. Flame the neck of the tube. Insert the loop into the broth culture and then withdraw it. The loop now holds inoculum. Replace the cap on this tube.
5. Remove the cap from the tube containing sterile TY broth and flame the neck. Insert the inoculating loop directly into the broth and gently shake it. Replace the cap.

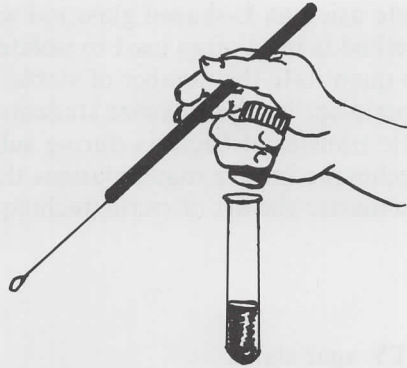


Figure 2.2. To remove a cap from a tube or bottle, grasp it with the fourth and pinky fingers. It should not touch any other surface before it is replaced.

6. Flame the inoculating loop a final time before putting it down.
7. Incubate the inoculated broth at 37° C overnight. This temperature is used as the incubation temperature because it optimizes the growth of *E. coli*, which is a normal inhabitant of the human intestinal tract.

B. Subculturing for Isolated Colonies—the Streak-Plate Technique

This technique is a rapid qualitative method used in the isolation of discrete bacterial colonies from a dense culture. There are many different means of preparing a streak plate. One commonly used method is described below.

1. Aseptically remove a loopful of inoculum from the overnight *E. coli* culture.
2. Holding the inoculating loop as you would a pen, touch the loop to the surface of a TY agar plate near the edge of the agar. Lightly streak the inoculum back and forth across the plate, until approximately one quarter of the plate has been covered.
3. Flame the inoculating loop and cool. Streak through the first area two or three times, then streak the loop back and forth along the side of the plate several times.
4. Flame the loop and cool once again. Streak through the second area two or three times, then along the side of the plate as before. Streak one last time into the center of the plate. (Refer to figure 2.3 for an illustration.)
5. Incubate the plate in an inverted position at 37° C overnight. The bacterial colonies found growing at the end of the streak should be completely isolated from each other. If this does not occur, repeat the streak plate until isolated colonies are obtained.

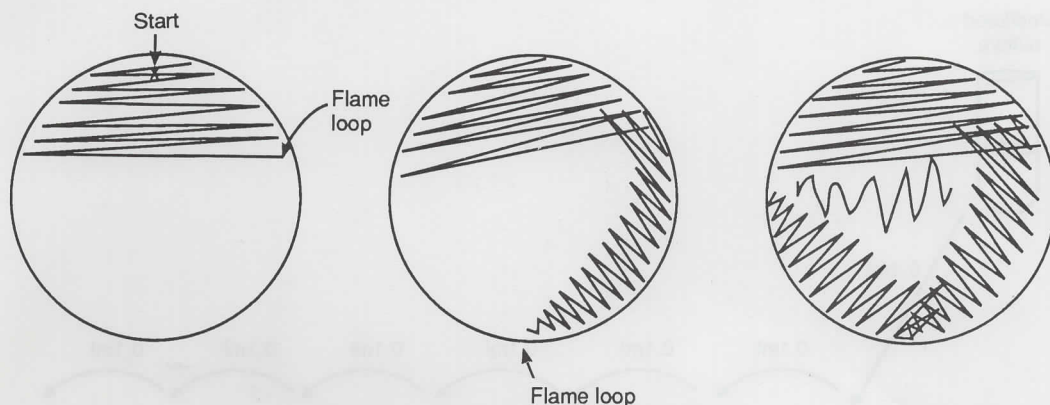


Figure 2.3. Preparation of a streak plate for the isolation of discrete bacterial colonies.

C. Preparation of a Pure Culture

It is often necessary for molecular biologists to prepare a pure culture of a bacterial strain that contains a gene of interest. The first step in this process is to streak for isolation, as described above. Isolated colonies can then be transferred to storage media for long- or short-term storage. The easiest means of storing a culture for a short time (three to four months) is to subculture one pure colony to slant medium in a tube that can be tightly capped. This keeps the bacterial cells in contact with nutrients; tightening the cap prevents the storage medium from drying out.

1. Sterilize an inoculating loop and cool. Touch *one* isolated colony from the streak plate prepared in part B above with the loop.
2. Aseptically remove the cap from a tube of slant medium and flame the neck.
3. Inoculate the slant by inserting the loop into the tube and placing it at the bottom of the agar slant. Then draw the loop upward, streaking the inoculum onto the surface of the medium.
4. Reflame the neck of the slant tube and replace the cap. Tighten it slightly to allow air to circulate.
5. Incubate at 37° C overnight. Once the pure culture has grown to sufficient density, tighten the cap and place it at 4° C. This arrests the growth of the culture. It can be stored for approximately three months on the slant, and can be used as a source of viable *E. coli* C600 by simply subculturing it to new media.

D. Determination of Cell Density

It is often necessary to quantitate the number of viable cells in a bacterial culture. One method of accomplishing this is to perform serial dilutions on a dense culture and plating the dilutions on agar plates. After the plates are incubated, colonies will

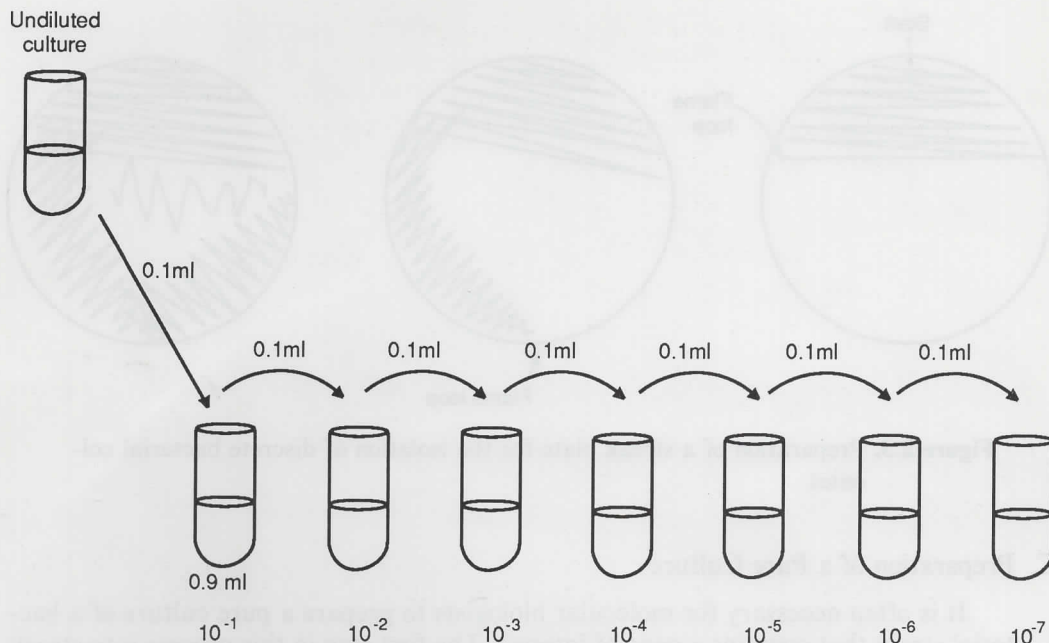


Figure 2.4. Serial 10-fold dilutions of a concentrated bacterial culture.

form from each individual viable bacterial cell, or *colony forming unit (cfu)*. The colonies can then be enumerated to provide an estimate of the cell density of the original culture.

- Using aseptic technique, prepare 10-fold serial dilutions from the overnight *E. coli* broth culture until the culture is diluted to 10^{-7} , as follows:
 - Dispense 0.9 ml of sterile water into seven sterile dilution tubes. Add 0.1 ml of culture to the first tube and mix completely.
 - Remove 0.1 ml from the first dilution, and add it to the second tube. Mix. Continue diluting in this manner until the culture has been diluted to 10^{-7} , as shown in figure 2.4.
- Using a sterile 1-ml serological pipette, remove 0.1 ml from the 10^{-4} dilution, and add it to the center of a TY agar plate.
- Sterilize a glass spreader by first submersing it in 95% ethanol. Quickly pass the spreader through the flame of a Bunsen burner, and allow the ethanol to burn off. Cool the spreader for ten seconds.
- Use the sterile spreader to spread the bacterial solution uniformly over the surface of the plate. This can be most efficiently accomplished by placing the petri dish on a small turntable and moving the spreader back and forth over the surface of the medium, while turning the turntable with the other hand. Continue spreading until all of the liquid has been absorbed onto the plate.
- Repeat with 0.1 ml of the 10^{-5} and 10^{-6} dilutions on new plates.

6. Incubate the plates in an inverted position at 37° C overnight.
7. Count the number of colonies that grow on the plates. To insure statistical accuracy, plates with more than 300 colonies or less than 30 colonies should not be counted. The concentration of viable cells in the original culture can be determined using the equation below:

$$\text{cfu/ml} = \text{number of colonies} \times \text{dilution factor}$$

For example, if 50 colonies grew on the 10^{-5} dilution plate, the concentration of viable cells in the original culture is:

$$50 \text{ colonies} \times 10^6 = 50 \times 10^6, \text{ or } 5 \times 10^7 \text{ cfu/ml}$$

The dilution factor in this case is 10^6 because 0.1 ml of the 10^{-5} dilution were plated, making the final dilution $10^{-5} \times 10^{-1}$, or 10^{-6} .

Assignment

After twenty-four hours:

- A. Examine the inoculated broth for growth. If the subculture was successful, the broth will take on a cloudy, or turbid, appearance because of the presence of millions of bacterial cells.
- B. Examine the streak plate for growth and for the presence of discrete, isolated colonies. If isolated colonies were not obtained in the first attempt, repeat the streak plate until they are.
- C. Prepare a pure culture from one of the isolated colonies on the streak plate by subculturing it to a TY agar slant.
- D. Count the number of colonies on the spread plates, and determine the cell density of the original *E. coli* culture in cfu/ml.

After forty-eight hours:

Examine the slant media for growth of a pure culture. Label it "stock *E. coli* C600" with the date, and store it at 4°C for use in following experiments.

Suggested Readings

- Gerhardt, P.; Murray, R.; Costilow, R.; Nester, E.; Wood, W.; Krieg, N.; and Phillips, G. 1981. *Manual of Methods for General Bacteriology*. American Society for Microbiology, Washington, DC.
- Stanier, R.; Ingraham, J.; Wheelis, M.; and Painter, P. 1986. *The Microbial World*, 5th ed. Prentice-Hall, New Jersey. 16–42.

NOTES AND CALCULATIONS

Chapter 3

Introduction to Bacteriophage λ

Viruses are obligate intracellular parasites incapable of metabolism or reproduction without a host cell. Bacteriophage lambda (λ) is an extensively studied bacteriophage that infects *E. coli*. The life cycle of λ consists of infection and replication, and then proceeds through one of two developmental phases, the *lytic cycle* or *lysogenic cycle*. λ first attaches to the bacterial host cell and injects its genetic information. The infected bacterium's replicative and biosynthetic machinery is then subverted to reproduce the phage genome and to synthesize phage-encoded proteins. In a short time, the virus reproduces itself over 100-fold. Once the phage progeny are assembled, phage-encoded proteins lyse the cell wall of the infected bacterium, liberating new infectious progeny that are able to perpetuate the viral life cycle by infecting neighboring bacterial cells. This process is illustrated in figure 3.1.

The lysogenic, or temperate, life cycle of λ is different from the lytic cycle in that the phage enters into a relatively stable, long-term relationship with the host cell by controlling its replicative and lytic functions. To achieve this, the DNA from the bacteriophage becomes integrated into the host cell's genome via a localized integrative recombination event. The genes of the lytic pathway are repressed, and λ is stably maintained and replicated along with the host cell's genome. This stable relationship will be terminated if damage is inflicted upon the host cell. Damage to host DNA results in the bacteriophage "jumping ship" and entering the lytic pathway to escape from the dying cell, as illustrated in figure 3.2.

The presence of a lytic λ infection can be easily detected. λ produces small plaques in a lawn of host bacterial cells. Susceptible cells are incubated with a diluted phage culture in the presence of magnesium ions. During this incubation, the phage attaches itself to the host cell surface. Then the mixture is plated on a nutrient agar plate. Each bacterial cell will grow into a colony. Because there are so many colonies, a uniform, turbid growth of bacteria will form on the surface of the agar. At the same time that the lawn is growing, each of the phage particles present will infect and lyse a single bacterium, releasing about 100 progeny. Each of these progeny can infect neighboring bacteria. The cycle continues until the host bacterial population moves into the stationary phase of its growth cycle. A cleared area, or *plaque*, forms in the bacterial lawn, and represents the infection of one bacterial cell with a single phage, or *plaque-forming unit*. The size of the plaque is dependent upon the type of phage, the bacterial host used,

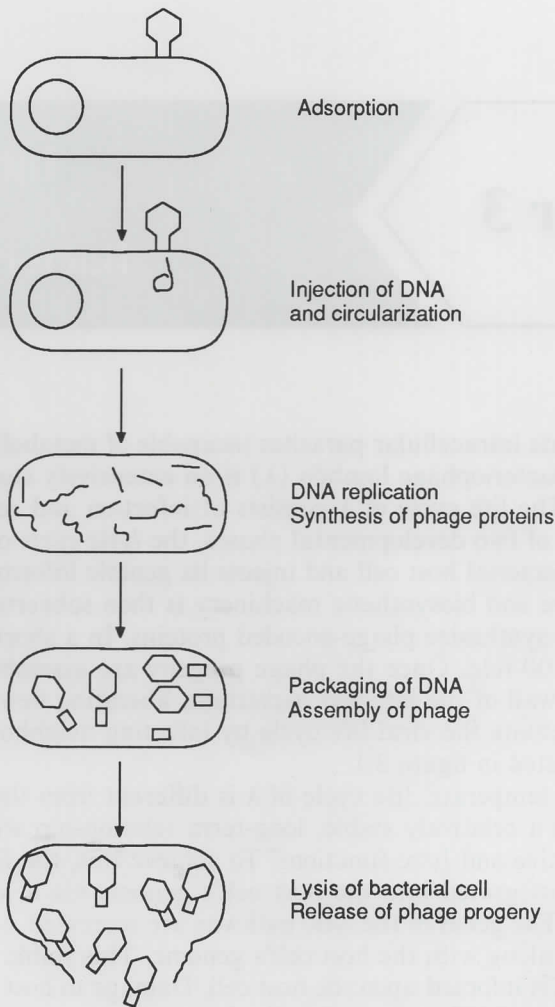


Figure 3.1. The lytic pathway of bacteriophage λ development.

and the conditions of plating and incubation. The *titer*, or concentration of a phage suspension, can be determined by diluting the phage stock and plating with host bacteria. The number of phage is related to the dilution factor of the stock and the number of plaques formed after incubation.

$$\text{pfu/ml} = \text{number of plaques} \times \text{dilution factor}$$

Bacteria that enter into a lysogenic state with a bacteriophage are termed *lysogens*. Lysogens carry integrated phage DNA in their genomes, which makes them resistant to infection by other phages. λ in the lysogenic state encodes a repressor protein, *cI*, that represses the lytic pathway by repressing expression of nearly all λ genes. λ will

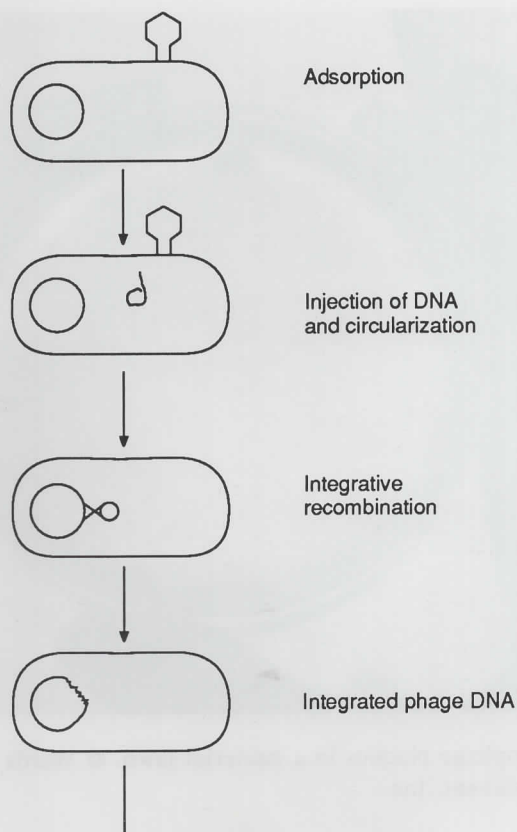


Figure 3.2. The lysogenic pathway of bacteriophage λ development.

only enter into the lytic cycle from lysogeny if the cI repressor becomes inactive. A temperature-sensitive mutation in the cI gene, cI_{857} , results in inactivation of the cI repressor at temperatures above 37°C . This mutation has been found to be a convenient means of inducing the lytic cycle, and strains of λ that have this mutation are frequently used in research laboratories.

Bacteriophage λ has been extensively used in molecular biology studies for two reasons. First, it is an interesting genetic system to study. Second, it has become an important vector for cloning experiments in which the foreign DNA to be cloned is from a higher eukaryote. Large pieces of foreign DNA can be cloned using λ , which is important because most eukaryotic genes are very long. Because of its usefulness in molecular biology, it is important to master the basic techniques of phage manipulation.

Materials

Media

TY broth; TB top agar; TY agar plates; TY + 0.1% maltose

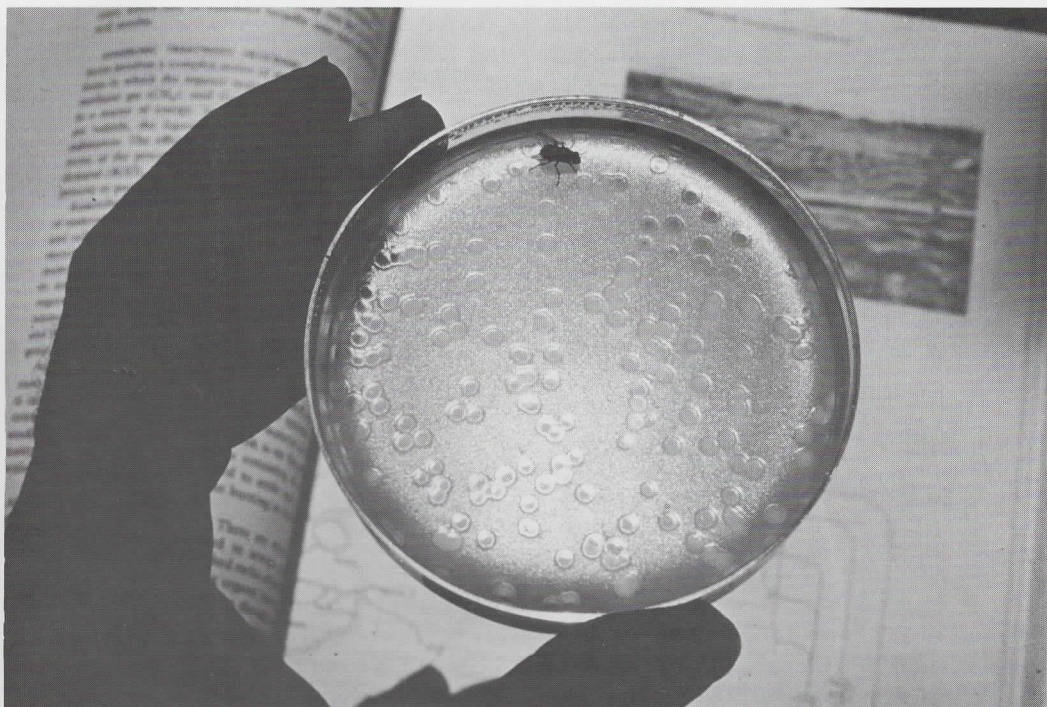


Figure 3.3. Bacteriophage plaques in a bacterial lawn. © Wards Natural Science Establishment, Inc.

Reagents

0.01 M MgSO_4

Supplies

1-, 5-, and 10-ml sterile serologic pipettes; sterile dilution tubes; 13 × 100 sterile test tubes

Cultures

E. coli C600 stock; *E. coli* C600(λcI_{857}) lysogen; λ stock culture

Procedure

Twenty-four hours before the scheduled lab period, prepare cultures for use in this experiment. Inoculate 5 ml of TY broth with *E. coli* C600 and incubate overnight at 37° C. Also, inoculate 5 ml of TY broth with *E. coli* C600(λcI_{857}). Incubate this culture overnight at 35° C.

A. Induction of Lysogen

1. Remove 1 ml of lysogen from the overnight culture and add it to a sterile test tube. Incubate this tube at 42° C for one hour. During this incubation, λ will become induced and will enter the lytic pathway.

B. Preparation of Plating Bacteria

1. Centrifuge the *E. coli* C600 culture and the remaining lysogen for 5 minutes at 10,000 rpm. The bacteria will form a pellet on the bottom of the tube.
2. Pour off the supernatant and discard it. Resuspend the pellets by adding 5 ml of 0.01 M MgSO_4 and vortexing until a uniform suspension is achieved. Place the plating bacteria on ice. At this point, plating bacteria can be stored for up to a week at 4° C.

C. Phage Titering

1. Microwave a bottle of TB top agar and hold it at 48° C.
2. Serially dilute the phage stock culture, as shown below, in TY + 0.1% maltose broth.

Tube:	1	2	3	4	5	6	7
Dilute:	1:100	1:100	1:10	1:10	1:10	1:10	1:10
Dilution:	10^{-2}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}	10^{-9}

To prepare a 1:100 dilution, add 0.01 ml (10 μl) of culture to 0.99 ml of sterile broth. To prepare a 1:10 dilution, add 0.1 ml of culture to 0.9 ml of broth.

3. Add 0.1 ml of *E. coli* C600 plating bacteria to 5 sterile test tubes. Add 0.1 ml of diluted phage from dilution tubes 3 through 7 and gently mix.
4. Repeat using lysogen as plating bacteria.
5. Incubate the bacteriophage and bacteria at 35°C for 10 minutes. During this incubation, the phage will adsorb to the bacterial cell surface.
6. Add 2.5 ml of molten TB top agar to each tube. Mix the contents by briefly rolling the tube between your hands, and then immediately pour the mixture over the surface of a TY agar plate. Rotate the plate once or twice to cover uniformly the agar surface. In order to prevent the top agar from hardening, prepare and pour only one tube at a time.
7. Allow the top agar to harden completely, then incubate the plates overnight in an inverted position at 35° C.

D. Titration of Induced Lysogen

1. Centrifuge the induced lysogen from part A for 10 minutes at 10,000 rpm. Remove the supernatant, which contains phage.
2. Prepare serial dilutions of the induced lysogen exactly as described in part C above. Plate dilutions 3 through 7 using *E. coli* C600 as plating bacteria. Allow the plates to harden, and incubate overnight at 37° C.

E. Determination of Viable Phage Concentration:

1. Count the dilution plates that contain between 30 and 300 plaques.
2. Calculate the titer of the original phage stock in pfu/ml:
 - a. when *E. coli* C600 was used as the plating bacteria.
 - b. when *E. coli* C600(λ C1857) was used as the plating bacteria.
3. Calculate the titer of the induced lysogen.

Assignment

After 24 hours:

1. Count the number of plaques that appear on the dilution plates, and determine the phage titer when *E. coli* C600 and C600(λ C1857) were used as the plating bacteria. Also, determine the titer of phage from the induced lysogen.
2. Compare the titer of phage found when λ was plated on wild type versus lysogen *E. coli*. What do these results indicate?
3. Calculate the concentration of phage in the stock λ culture in pfu/ml.

Suggested Readings

- Hendrix, R.; Roberts, J.; Stahl, F.; and Weisberg, R., eds. 1983. *Lambda II*. Cold Spring Harbor Laboratory, New York.
- Ptashne, M. 1986. *A Genetic Switch: Gene Control and Phage λ* . Cell Press and Blackwell Scientific Publications, Massachusetts and California.

NOTES AND CALCULATIONS

Chapter 4

Gene Cloning I: Making a Recombinant Plasmid

Introduction

Plasmids are small, circular molecules of DNA found in bacterial cells. These extrachromosomal bits of double-stranded DNA are found in a wide range of bacterial species, and can be very small (less than 2,000 base pairs in length), or very large (over 100,000 base pairs in length). Plasmids encode genes that are not absolutely required for host cell function, such as genes for resistance to antibiotics, or for transmission of the plasmid to genetically similar bacteria. These incredible molecules have become very important to molecular biologists in recent years because they can be easily extracted from bacterial cells and can be manipulated genetically.

All bacterial plasmids carry genes that allow them to replicate autonomously within a bacterial cell. When a single plasmid enters a susceptible host cell, it replicates until its characteristic copy number is reached. The copy number, or number of plasmids per bacterial cell, is dependent upon the genetic constitution of both the plasmid and the cell; it ranges from one copy up to two hundred copies per cell. Once the correct copy number is reached, replication of the plasmid DNA is shut off, and the plasmids are maintained within the cell. When a bacterial cell containing a plasmid divides, the plasmid is stably inherited, so that each daughter cell receives at least one plasmid. In this way, plasmid molecules are propagated in the bacterial population.

Plasmids are made of double-stranded DNA, and therefore contain restriction endonuclease recognition sites. Restriction endonucleases cleave DNA only at specific recognition sequences, which range from four to eight base pairs in length. These enzymes are readily available commercially. Restriction reactions *in vitro* have strict environmental requirements that differ among enzymes. These requirements include the salt concentration of the reaction buffer, the pH at which the reaction is carried out, and the incubation temperature. Most manufacturers of restriction enzymes supply researchers with the information necessary to optimize restriction reactions (see appendix III).

Restriction enzymes have the unique property of making double-stranded breaks in DNA at *palindromic sequences*, regions that exhibit two-fold symmetry about a given axis. When a molecule of DNA such as a plasmid is cut with restriction endonucleases, a number of "restriction fragments" are generated. This process is similar to cutting a circular piece of string with scissors. If the string is cut once, a long, linear fragment is obtained that represents the full length of the circle. Cutting the string more than once

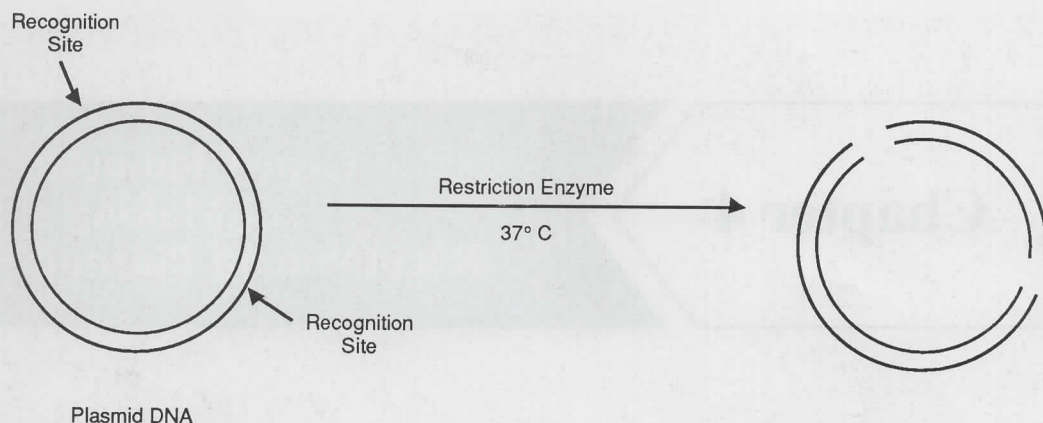


Figure 4.1. Restriction endonucleases cleave double-stranded DNA to yield fragments of variable size. Any given endonuclease will cleave DNA every 4^n base pairs in a segment of DNA, where four is the number of possible bases (ACGT), and n is the number of bases in the recognition sequence. For example, the recognition sequence for EcoR I is six bases long. Therefore, EcoR I should cut every 4^6 , or 4,096 base pairs, in a piece of DNA.

yields a number of shorter pieces, whose sizes represent the distance between each of the sites cut with the scissors. When this is done with plasmid DNA, the sizes of the fragments represent the distance between restriction sites, as illustrated in figure 4.1. Breaking molecules of DNA into smaller pieces provides scientists with the ability to work with smaller pieces of DNA, sometimes single genes.

Restriction fragments can be viewed by agarose gel electrophoresis. Samples of DNA are placed in wells cut into a horizontal slab of agarose and are electrophoresed. Heated molten agarose, when cooled, solidifies to form a solid matrix through which macromolecules can migrate. DNA molecules are uniformly negatively charged and will travel toward the anode in an applied electrical field. Therefore, DNA molecules are separated in an agarose gel on the basis of size. Larger molecules migrate slowly through the matrix, while smaller molecules slither through the meshwork created by the agarose and travel farther toward the anode. The gel is stained with *ethidium bromide*, a fluorescent agent that intercalates between the bases of DNA. When the gel is placed on an ultraviolet light source, the DNA becomes visible as light bands on the dark background of the gel. DNA standards of known molecular size are electrophoresed along with the samples to provide a size comparison (see figure 4.2).

Restriction endonucleases cleave DNA at palindromic sequences to leave either blunt or cohesive ends. Blunt ends result when cleavage occurs in the middle of a palindromic sequence. Cohesive, or sticky, ends result when the restriction enzyme makes staggered cuts within its recognition sequence, leaving single-stranded ends, as shown in figure 4.3. Fragments that possess complimentary cohesive ends can be joined with the help of DNA

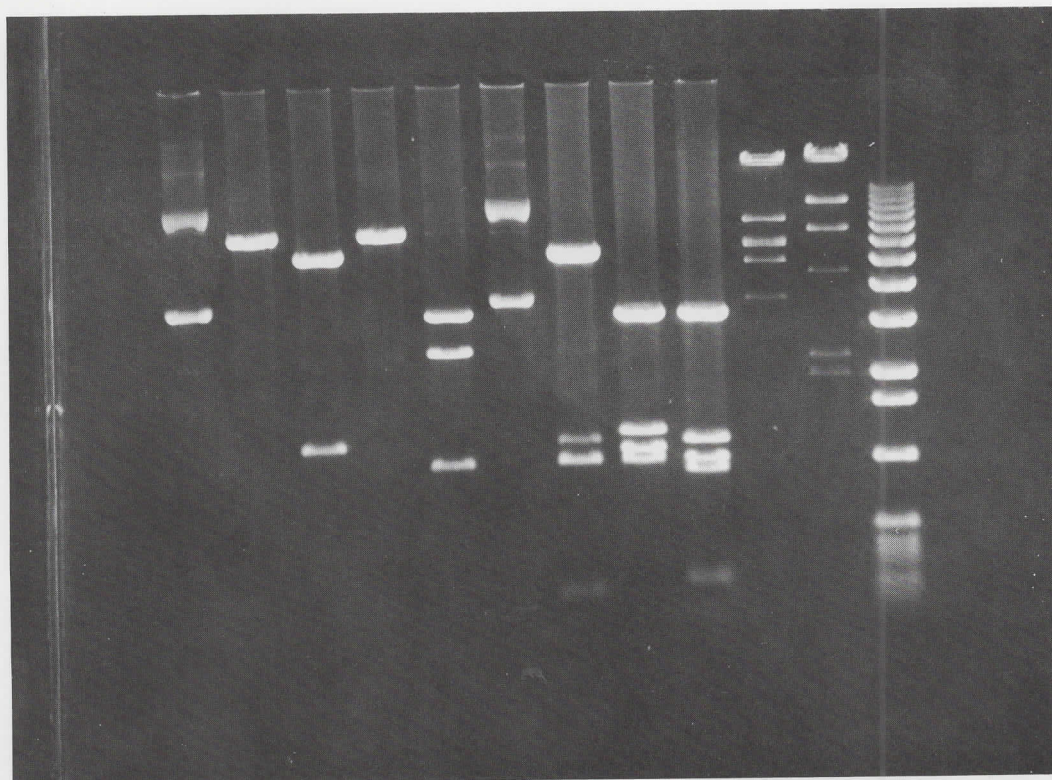


Figure 4.2. Restriction fragments stained with ethidium bromide and viewed under ultraviolet light.

ligase, that covalently links the 3'-OH of one nucleotide to the 5'-P on the adjoining one. This creates a recombinant molecule made up of two pieces of DNA derived from different sources. A plasmid that has been cut with a given restriction enzyme can thus be joined to DNA fragments from almost any source, provided that it was cut with the same restriction enzyme. This recombinant plasmid can be transformed into a bacterial host cell, which then becomes the source of an abundant supply of the plasmid and, by extension, of the foreign DNA.

Some restriction enzymes make cuts that are similar or identical to those produced by other restriction enzymes. *Isochizomers* are unrelated restriction enzymes that recognize the same sequence in DNA. Some isochizomers cleave in exactly the same way, while others make different cuts, leaving incompatible sticky ends. Some restriction enzymes with different recognition sequences can cleave DNA and generate compatible sticky ends, such as BamH I and Bgl II. BamH I recognizes and cleaves the sequence 5'-G GATCG-3'. Bgl II cleaves 5'-A GATCT-3'. The cohesive ends generated by this cleavage are identical, and can base pair in a complimentary fashion. However, this creates a hybrid restriction site, which cannot be cleaved again by either BamH I or Bgl II.

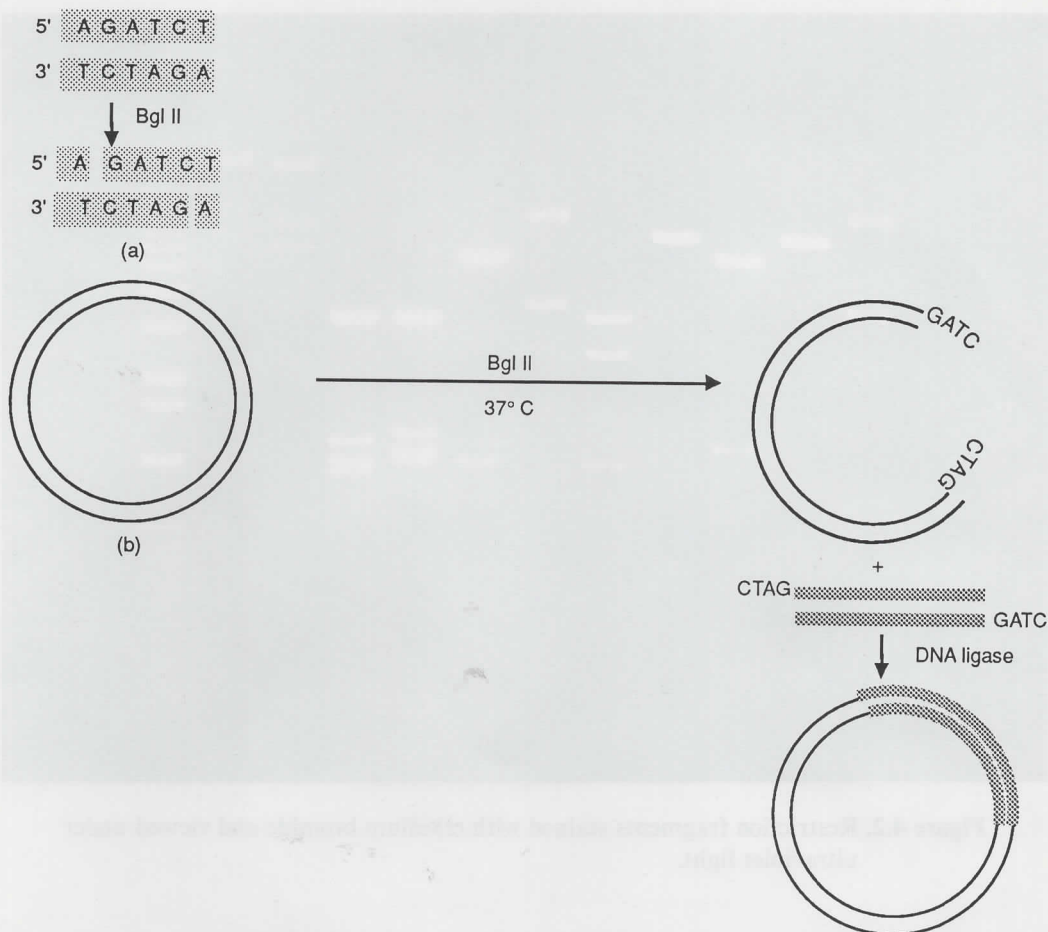


Figure 4.3. a. Cutting DNA with Bgl II yields cohesive ends with 4 base overhangs.
 b. A recombinant plasmid is made by cutting plasmid and foreign DNA with the same restriction enzyme generating compatible cohesive ends. The compatible ends anneal and are completely sealed with DNA ligase.

The plasmid pBR322, shown in figure 4.4, is often used in cloning experiments because of its versatility. It is a small, autonomously replicating, circular DNA molecule, that is maintained at 5–10 copies per *E. coli* cell. pBR322 has two antibiotic resistance genes, ampicillin resistance (Amp^R) and tetracycline resistance (Tet^R).

Within the sequence of the ampicillin resistance gene are unique recognition sequences for the restriction enzymes Pst I and Pvu I. Foreign DNA can be inserted into either one of these two sites by simply cutting the plasmid, adding foreign DNA with complimentary sticky ends, and ligating the two DNAs together. Insertion of the foreign DNA results in *insertional inactivation* of the ampicillin resistance gene. To screen for recombinant molecules, transformed bacteria are replica-plated on medium supple-

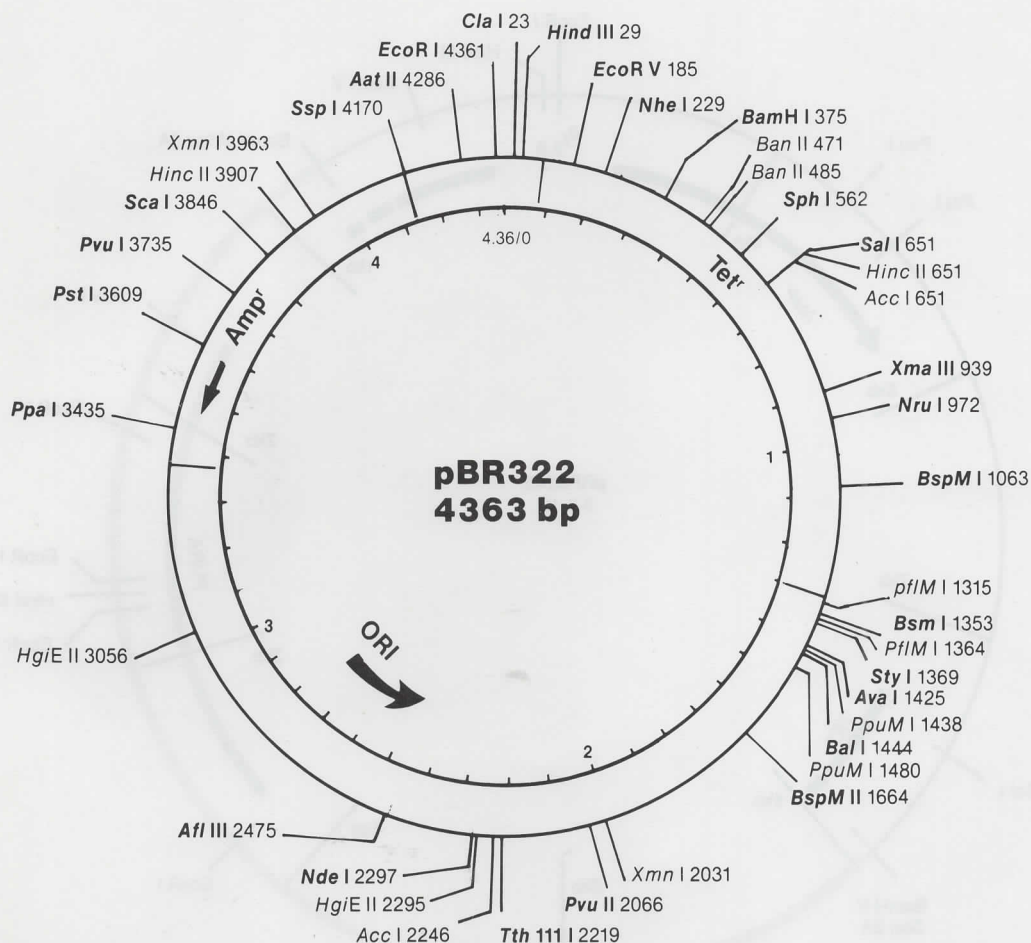


Figure 4.4. Restriction map of pBR322. From *Stratagene 1984 Product Catalog*. Copyright © 1989 Stratagene, LaJolla, CA.

mented with the antibiotics ampicillin and tetracycline. Cells carrying the recombinant molecule will form colonies on the tetracycline-supplemented medium, but will be unable to grow in the presence of ampicillin. The *Bam*H I and *Sal* I sites found within the tetracycline gene sequence can be used in a similar manner.

In this experiment, the *purA* gene from *E. coli* will be subcloned from the plasmid pRPC245 (a pBR322 derivative) into the tetracycline resistance gene of pBR322. The restriction map for pRPC245 is shown in figure 4.5. The region between the *Nde* I and *Sal* I sites on pBR322 will be removed following a digestion with both enzymes. A fragment containing the *purA* gene, approximately 3.0 kb in length, will be ligated into this site as shown in figure 4.6. This recombinant molecule will later be transformed into *E. coli* and stored for use in subsequent experiments.

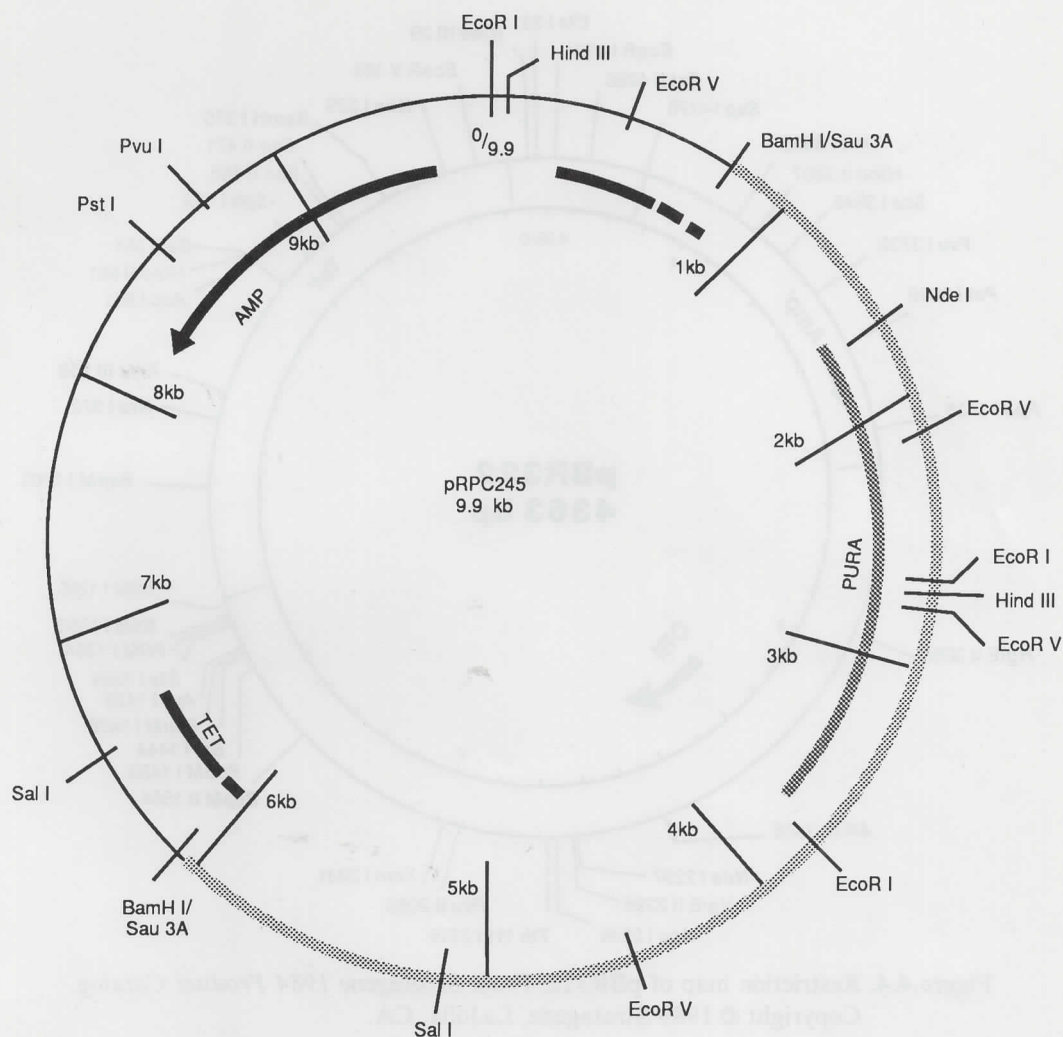


Figure 4.5. A partial restriction map of pRPC245, showing the approximate placement of restriction sites.

Materials

Reagents

Purified pRPC245 DNA; purified pBR322 DNA; Nde I and 10X reaction buffer; Sal I and 10X reaction buffer; Hind III and 10X reaction buffer; T4 DNA Ligase and 10X reaction buffer (for enzymes and reaction buffers refer to accompanying manufacturer's instructions); TE pH 8.0; electrophoresis grade agarose; 50X TAE; ethidium bromide (10 mg/ml); 6X Sample Buffer; phenol; Sevag (24 parts chloroform:1 part isoamyl alcohol); 5 M NaCl; 95% ethanol; 70% ethanol; sterile distilled water.

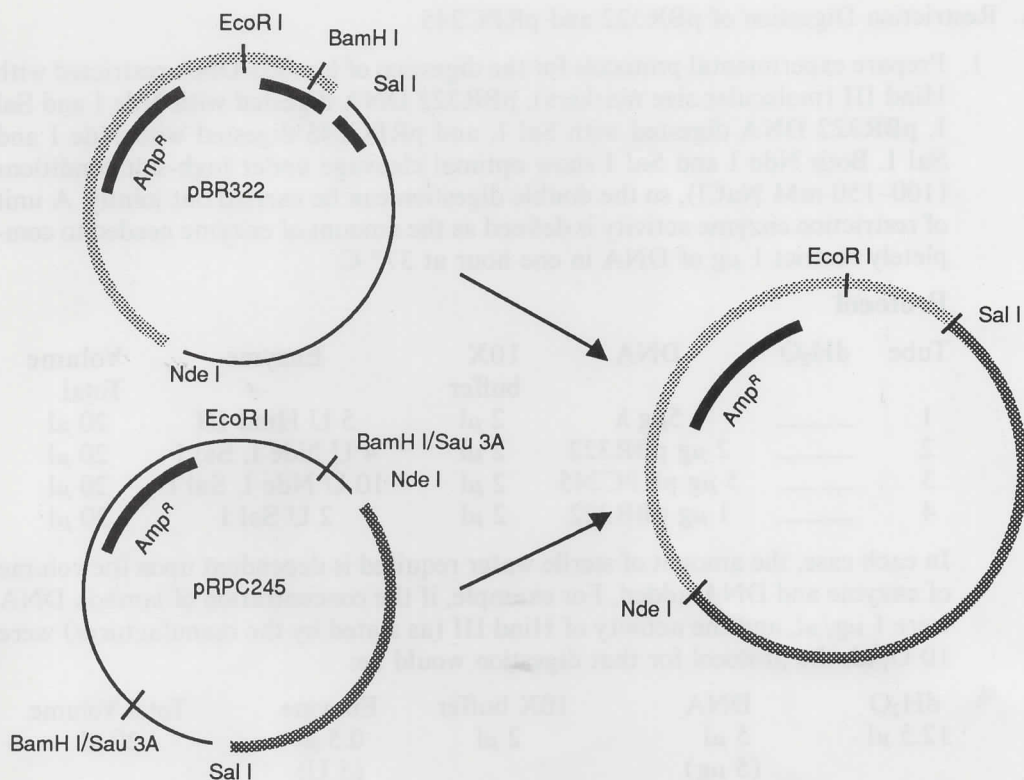


Figure 4.6. Construction of a subclone containing the *E. coli purA* gene.

Equipment and Supplies

Mini-gel electrophoresis apparatus; power supply; microcentrifuge; microcentrifuge tubes; micropipettor capable of 1–5 μ l or glass microcapillary tubes; sterile micropipette tips; Teflon spatula; vortex; UV light box; 37° C waterbath; –70° C freezer or an ethanol-dry ice bath.

Procedure

Note:

1. Restriction enzymes and plasmid DNA are precious commodities and must be treated with respect. The enzymes, their buffers, and the DNA must be kept on ice at all times.
2. Ethidium bromide is used to stain DNA because it intercalates between base pairs and is visible under ultraviolet light. Intercalation results in a distortion of the DNA double helix, and *in vivo* is mutagenic. Wear gloves and use caution when working with this chemical.

A. Restriction Digestion of pBR322 and pRPC245

1. Prepare experimental protocols for the digestion of lambda DNA restricted with Hind III (molecular size markers), pBR322 DNA digested with Nde I and Sal I, pBR322 DNA digested with Sal I, and pRPC245 digested with Nde I and Sal I. Both Nde I and Sal I show optimal cleavage under high-salt conditions (100–150 mM NaCl), so the double digestion can be carried out jointly. A unit of restriction enzyme activity is defined as the amount of enzyme needed to completely restrict 1 μ g of DNA in one hour at 37° C.

Protocol

Tube	dH ₂ O	DNA	10X buffer	Enzyme	Volume Total
1	_____	5 μ g λ	2 μ l	5 U Hind III	20 μ l
2	_____	2 μ g pBR322	2 μ l	4 U Nde I, Sal I	20 μ l
3	_____	5 μ g pRPC245	2 μ l	10 U Nde I, Sal I	20 μ l
4	_____	1 μ g pBR322	2 μ l	2 U Sal I	20 μ l

In each case, the amount of sterile water required is dependent upon the volume of enzyme and DNA added. For example, if the concentration of lambda DNA were 1 μ g/ μ l, and the activity of Hind III (as stated by the manufacturer) were 10 U/ μ l, the protocol for that digestion would be:

dH ₂ O	DNA	10X buffer	Enzyme	Total Volume
12.5 μ l	5 μ l (5 μ g)	2 μ l	0.5 μ l (5 U)	20 μ l

Complete the protocol for tubes 2, 3, and 4.

2. Add the reactants as determined to autoclaved microcentrifuge tubes in the following order: water, buffer, DNA, and enzyme. Mix the contents, and then briefly spin the tubes in a microfuge (one or two seconds at high speed).
3. Incubate the tubes in a 37° C waterbath for one to two hours.

B. Preparation of a 0.7% Agarose Mini-gel

1. In a 200-ml flask, mix 0.35 g of agarose with 52 ml of distilled water. Heat to boiling to dissolve agarose.
2. Cool the solution slightly and add 1 ml of 50X TAE. Carefully add 2.5 μ l of 10 mg/ml ethidium bromide (*Caution:* mutagen) and gently swirl the solution to mix.
3. When the molten agarose has cooled to approximately 60° C, pour it into the casting tray of a mini-gel electrophoresis apparatus. Put the comb in place and allow the gel to solidify (see figure 4.7).
4. While the gel is solidifying, prepare 250 ml of running buffer (1X TAE). Add 5 ml of 50X TAE to 245 ml of distilled water. Add 12.5 μ l of ethidium bromide, and swirl gently to mix.
5. When the gel has hardened, carefully remove the comb to form sample wells in the agarose. Place the casting tray with gel into the electrophoresis apparatus.

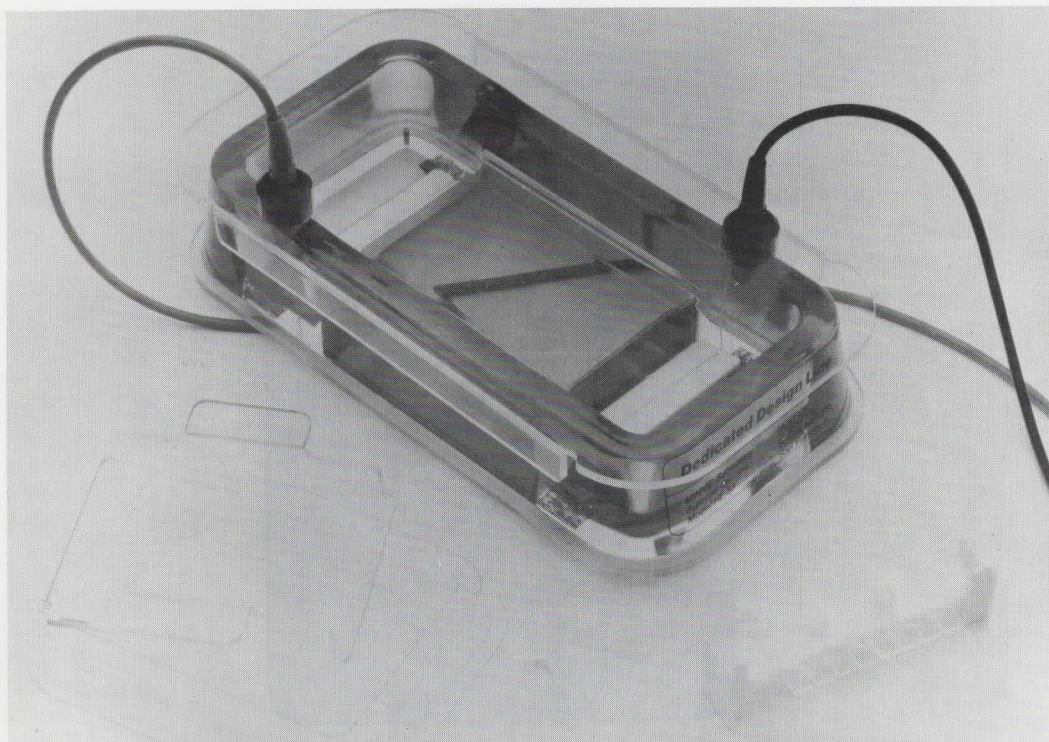


Figure 4.7. A horizontal submarine agarose mini-gel electrophoresis. Courtesy of Hoefer Scientific Instruments.

6. Add running buffer to the buffer chambers until the surface of the gel is submerged. The mini-gel is now ready to be loaded.

C. Loading the Mini-gel

1. After the restriction digestions from part A have incubated for approximately one hour, add 4 μ l of 6X Sample Buffer to each tube and mix. Spin each tube briefly in a microfuge to bring all of the sample to the bottom of the tube.
2. Using a micropipettor set at 24 μ l, carefully add each sample to the appropriate sample well in the agarose gel.
3. Electrophorese the samples at 100 volts (constant voltage) for approximately thirty minutes. The negatively charged DNA will migrate toward the anode and will separate according to size. The extent of migration can be monitored during electrophoresis by watching the progress of the two tracking dyes in the sample buffer, xylene cyanol and bromophenol blue.
4. Once appropriate migration has been achieved, disconnect the apparatus from the power supply, and place the gel directly on a UV light box. A photograph of the gel should be taken at this time. (Ultraviolet light is mutagenic. When working around UV, all exposed skin should be covered and protective eye covering should be worn.)

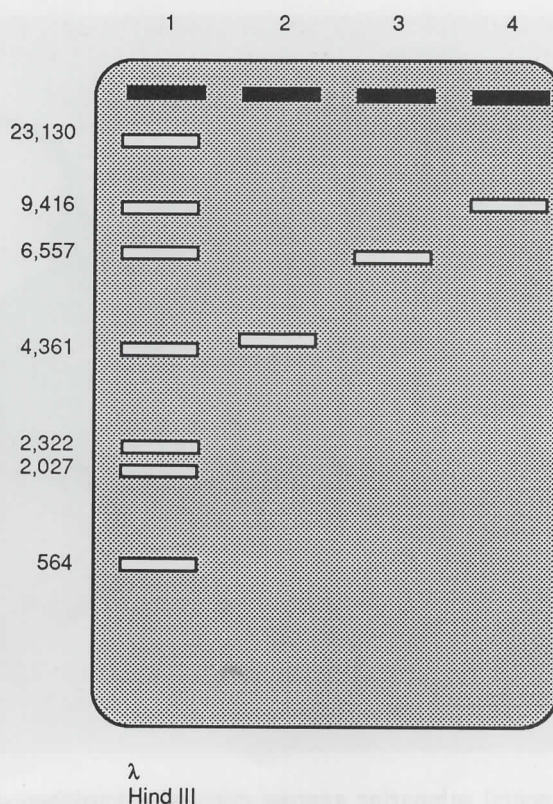


Figure 4.8. Sketch of an agarose gel showing a lambda DNA-Hind III digestion in lane 1, to generate molecular size standards of known size. Lanes 2, 3, and 4 contain DNA fragments of approximately 4.4 kb, 6.4 kb, and 9.4 kb, respectively.

D. Purification of DNA Fragments from an Agarose Gel

Digestion of lambda DNA with the restriction enzyme Hind III yields the DNA fragments shown in figure 4.8. These molecular size markers can be used to estimate the size of the fragments generated from the preparative digestions of pBR322 and pRPC245.

The size of the fragments generated following restriction of the plasmids with Nde I and Sal I can be predicted by examining the restriction maps of pBR322 and pRPC245 (refer to figures 4.4 and 4.5).

1. Place the gel on a UV light box. Compare the size of the fragments in the digested plasmid lanes to the molecular size standards, and determine which fragments to use in the subclone construction (from figures 4.4, 4.5, and 4.6).

2. Using a Teflon spatula, carefully make cuts in the agarose around the appropriate bands. Slice as close to the band as possible. The goal is to remove the DNA and leave most of the agarose behind.
3. Carefully remove the gel slices from the gel and place them both into a single microfuge tube.
4. Macerate the gel slices with the spatula.
5. Add 100 μl of phenol and vortex at high speed for one minute.
6. Place the tube at -70°C (or in an ethanol-dry ice bath) and freeze for fifteen minutes.
7. Centrifuge the tubes for five minutes in a microcentrifuge. The solution will separate into two phases: an upper aqueous phase that contains the DNA, and a lower organic phase that contains the phenol and agarose.
8. Carefully remove the upper aqueous phase and transfer it to a new microfuge tube. Using a micropipettor, measure the volume of the DNA solution.
9. Add an equal volume of phenol to the DNA solution and vortex for thirty seconds. Centrifuge for two minutes at high speed to separate the phases once again. Remove the upper aqueous phase and transfer it to a new tube. (This process is called an *extraction*.)
10. Repeat the extraction with a 1:1 mixture of phenol and Sevag. Remove the upper phase and transfer it to a new tube.
11. Repeat the extraction a final time with Sevag. Remove the upper phase and measure the volume of the DNA solution.

E. Ethanol Precipitation of DNA Fragments

1. Following the final extraction step, add one-tenth volume of 5 M NaCl to the DNA solution. For example, if the final volume of the DNA solution is 100 μl , add 10 μl of 5 M NaCl.
2. Add two volumes of ice cold 95% ethanol to the DNA/salt solution. (From the above example, add 220 μl ethanol.) Close the cap and mix the contents vigorously by rapidly inverting the tube several times.
3. Incubate the tubes at -70°C for fifteen to thirty minutes. At this point, if laboratory time is running short the tube can be stored for up to one week at either -70°C or -20°C and the ethanol precipitation can be completed at that time.
4. Centrifuge the tubes at 4°C for fifteen minutes at high speed (12,000 rpm). After this step, the DNA will form a white pellet at the bottom of the microfuge tube that may or may not be visible. Continue with the procedure even if a pellet is not seen.
5. Slowly pour off the supernatant. Briefly invert the tube over a paper towel and tap it once or twice to drain thoroughly the ethanol from the nucleic acid pellet.
6. Add 200 μl of 70% ethanol to the tube and close the cap. Flick the tube several times to wash the salt, which is soluble in 70% ethanol, from the pellet.
7. Centrifuge the tube at high speed for fifteen minutes at 4°C . Pour off the supernatant and invert the tube over a paper towel, tapping gently to drain off the ethanol.

8. Dry the pellet by blowing hot air, as from a blow dryer, over the top of the open tube. This will evaporate any remaining ethanol. It is important that all of the ethanol is evaporated and the DNA pellet is dry before continuing.
9. Add 24 μ l of TE buffer (pH 8.0) to the tube. Stand the tube at room temperature for five or ten minutes. Resuspend the pellet completely by flicking the tube or by slowly pipetting up and down with a micropipettor. DNA is stable in solution and at this point could be stored long term at 4° C or frozen.

F. Ligation of DNA Fragments to Create a Recombinant Plasmid

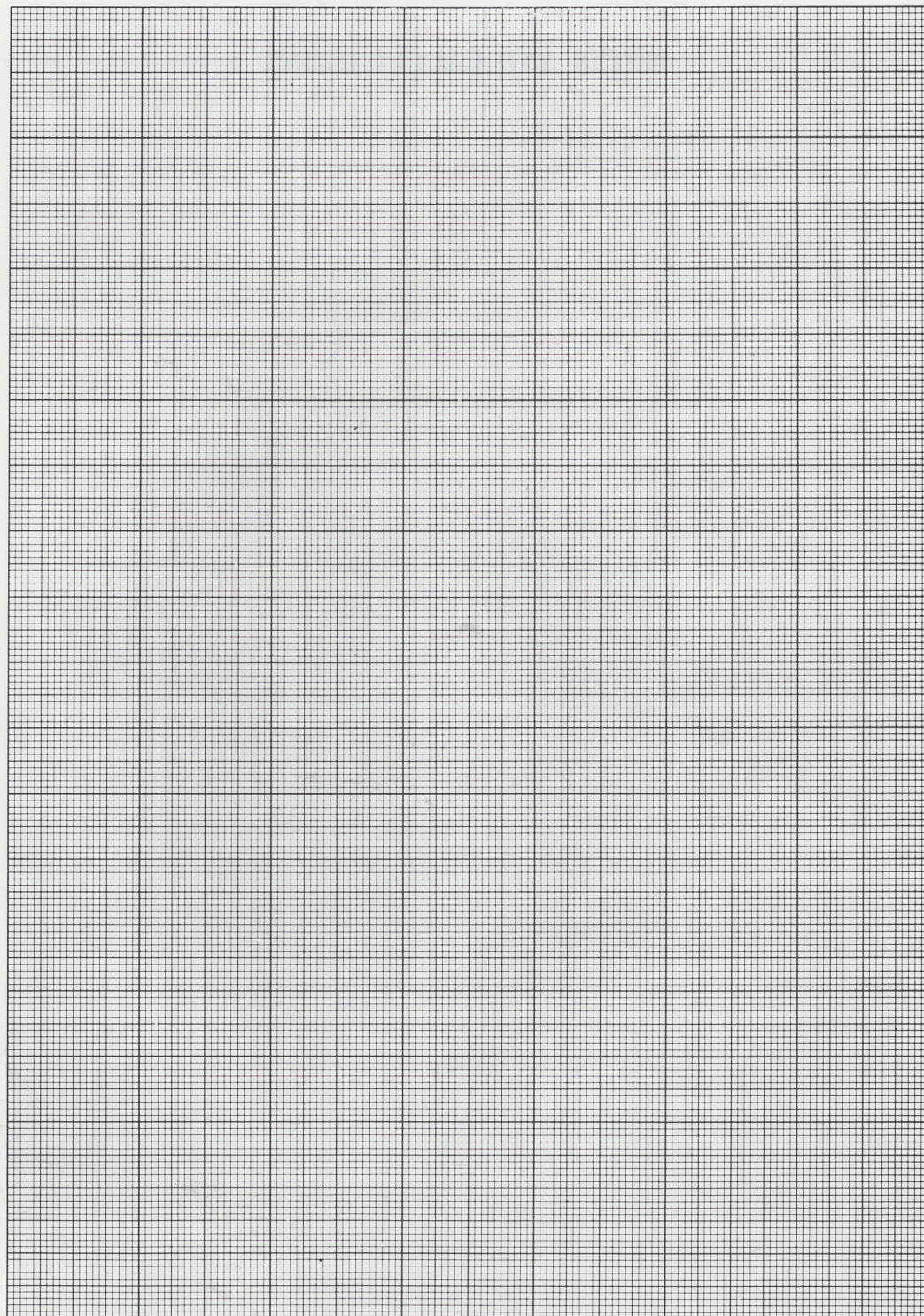
1. Set up the ligation reaction in a total volume of 20 μ l, as follows:
 - 16 μ l DNA solution
 - 2 μ l 10X ligase reaction buffer
 - 2 μ l T4 DNA ligase
2. Incubate the tube overnight in a 12° C waterbath. Label the newly formed recombinant molecule pMB1 (for Molecular Biology, plasmid 1). Store the plasmid at 4° C for later use.

Assignment

1. Prepare experimental protocols for the restriction enzyme digestions: λ -Hind III, pBR322-Nde I and Sal I, pRPC245-Nde I and Sal I, and pBR322-Sal I.
2. Determine the size of the DNA fragments generated by restriction of pBR322 and pRPC245.
 - a. From the photograph of the agarose gel, measure the distance (in cm) from the sample well to each band in the size standard (λ -Hind III) lane.
 - b. To prepare the standard curve, plot the given size of each λ -Hind III fragment (from figure 4.8) vs. 1/d on linear graph paper, and draw the best fit line through the data points.
 - c. To determine the molecular size of the restriction fragments, measure the distance to each band, calculate 1/d, and read the molecular size of each fragment directly from the standard curve.
3. Indicate on the photograph the fragments you used in the construction of the subclone. Describe the logic used in this determination.
4. Approximate the concentration of DNA in each restriction fragment by visually comparing the intensity of each band with the pBR322-Sal I digestion product (there is 1 μ g of DNA in this band). You are especially interested in the amount of DNA in the bands you used in your subclone construction.
5. Draw a picture of the newly constructed subclone, including the applicable restriction sites.

Suggested Readings

Sambrook, J.; Fritsch, E. F.; Maniatis, T. 1989. *Molecular Cloning, A Laboratory Manual, 2nd ed.* Cold Spring Harbor Laboratory Press, NY.



NOTES AND CALCULATIONS

Chapter 5

Gene Cloning II: Selection of a Cloned Gene

Introduction

The process of *transformation*, which is the uptake of DNA and its subsequent expression by a living cell, was originally observed by Frederick Griffith in 1928. In his experiments, he injected mice with heat-killed *Streptococcus pneumoniae*, a disease-causing (pathogenic) bacterium. Heat treatment rendered the bacterial cells inviable and unable to cause disease in mice. However, when Griffith mixed the heat-killed pathogenic bacteria with a nonpathogenic strain of *Streptococcus* and injected the mixture into mice, the mice became diseased and died. Thus, he observed that the heat-killed pathogenic bacteria transformed the nonpathogenic strain to make it pathogenic. This indicated that some kind of “factor” was physically transferred to the nonpathogenic bacteria, resulting in the expression of the pathogenic traits. Griffith’s “transforming factor” was eventually determined to be the genetic material DNA.

The natural process of transformation as originally described by Griffith occurs only rarely. With the molecular biology boom of the 1960s and 70s, attempts were made to make this process controllable and reproducible, to provide scientists with the opportunity to introduce foreign genes into living cells. It was found that the process of transformation had several requirements. Cells in the logarithmic growth phase were more likely to transform successfully DNA across their cell membranes than were stationary phase cells. Bacterial cells were found to have a transitory state of competence, a state in which cells were more likely to take up DNA. Competence is related to the conditions under which the DNA and cells are combined. Divalent cations, such as magnesium and calcium, are known to play an important role in the early stages of DNA uptake. Temperature is another important requirement. Cells and DNA interact more productively in a cold (0–5° C) environment. A brief heat shock at 42° C for one to two minutes following the cold incubation has also been shown to increase the *transformation efficiency* (defined as the number of transformants obtained from one microgram of DNA).

Transformation efficiency is also affected by characteristics of the transforming DNA. Small DNA molecules are transformed more efficiently than are larger ones. The efficiency is further increased if the DNA is in a circular form, such as intact plasmid DNA. Large, linear molecules are transformed at low efficiency and only with difficulty. Linear fragments transform at a low efficiency and are usually not stably maintained in bacterial cells because they are subject to attack by cellular nucleases.

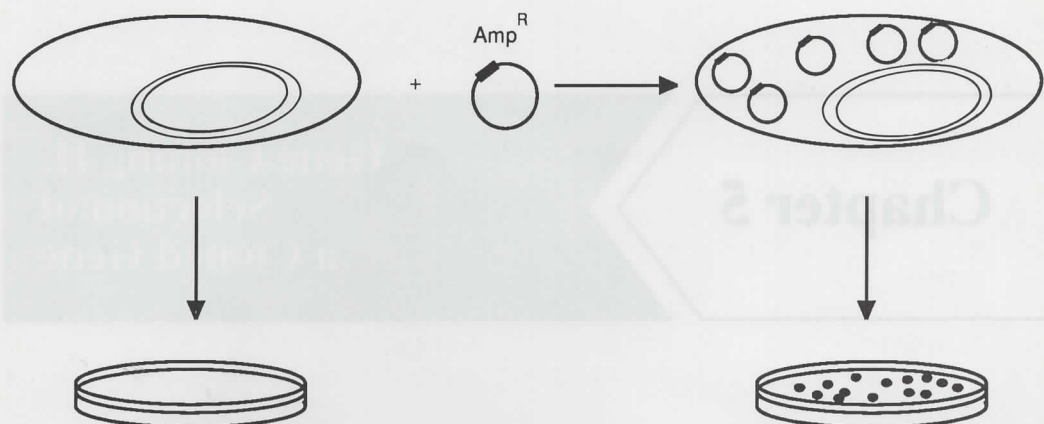


Figure 5.1. Transformation of wild-type *E. coli* strains with an antibiotic-resistant plasmid conveys resistance to the host cells.

Once the plasmid has been taken up across the bacterial cell membrane, it becomes established as a stable genetic element within the cell. Plasmids such as pBR322 and its derivatives will self-replicate until there is a characteristic number of plasmids per cell. The genes on the plasmid will be expressed by the host cell's machinery and thus, at least in the case of antibiotic resistance genes, can be detected phenotypically. Wild-type bacteria normally susceptible to antibiotics will express the antibiotic resistance genes encoded by the plasmid and will be rendered resistant. Thus, successful transformation of bacteria by plasmids carrying antibiotic resistance genes can be physically defined by the appearance of isolated colonies on selective (antibiotic-containing) medium. This process is illustrated in figure 5.1.

However, insertional inactivation of an antibiotic resistance gene does not guarantee the presence of a cloned gene in a plasmid. The antibiotic-resistant colonies should also be subjected to a genetic selection to fully characterize the recombinant plasmid. This can be done by determining which of the antibiotic-resistant clones show a phenotypic change from *auxotrophy* to *prototrophy* for the cloned trait. For example, pMB1 should carry the *E. coli* chromosomal gene *purA*. Cells mutant at the *purA* locus are adenine auxotrophs; that is, they must be supplied with adenine in order to grow. The plasmid pMB1, if it indeed carries the *purA* gene, should transform a mutant *E. coli purA*⁻ strain to prototrophy, or *purA*⁺. Once pMB1 is inside the mutant cells, they should be able to grow both in the presence and absence of adenine. Cells without pMB1, or cells carrying plasmids without the *purA* insert, will only grow when adenine is supplied in the media.

Whenever a recombinant plasmid carrying an *E. coli* gene is transformed into an *E. coli* host, it is wise to use a mutant *recA*⁻ host strain. The *recA* gene encodes the RecA protein in *E. coli*, which is involved in the process of homologous recombination. Recombination between the chromosomal gene and a homologous gene on the plasmid may occur if *recA*⁺ strains are used in transformation experiments. This effect is undesirable because the gene on the plasmid could become damaged or even lost. Using *recA*⁻ strains also prevents the formation of dimers or multimers between the copies of the plasmid within the cell.

In this experiment, *E. coli* strain HA3, an adenine auxotroph, will be transformed with the recombinant plasmid constructed in the previous experiment. The tetracycline resistance gene of pBR322 was inactivated by insertion of the foreign DNA fragment carrying the *purA* gene. Therefore, transformation with pMB1 will convey resistance to ampicillin, but not tetracycline, to the bacterial cell.

To determine which cells received the recombinant plasmid, ampicillin-resistant transformants will be replica-plated onto two types of minimal media plates, one supplemented with adenine and the other containing no supplements. Minimal medium contains only the minimum nutrients required to support the growth of wild-type *E. coli* (inorganic salts and a carbon source). Therefore, only the cells that were transformed with the plasmid carrying the *purA* gene will be able to grow on both types of medium. These cells can then be subcultured to new medium and stored as a source of pMB1 for future experiments.

Materials

Media

TY broth; TY-Amp agar plates; MM (minimal medium) plates; MM with adenine plates

Reagents

50X TAE; 10 mg/ml ethidium bromide; agarose; 100 mM CaCl_2 ; purified pBR322 DNA
1 $\mu\text{g}/\mu\text{l}$ stock solution

Equipment and Supplies

Mini-gel apparatus; power supply; turntable; glass spreader; 37° C water bath; 42° C waterbath; ice bucket with ice

Cultures

TY (adenine) agar plate culture of *E. coli* HA3 (*purA*⁻, *recA*⁻, *uvrA*⁻) with isolated colonies, grown for 24–36 hours at 37° C

Procedure

Before beginning the transformation procedure, it is important to determine the concentration of the pMB1 DNA obtained in the previous experiment. Competent cells become saturated at approximately 0.1–0.2 μg of DNA per ml of cells; an increase in plasmid concentration above this level, in fact, decreases the transformation efficiency.

Two methods are commonly used to determine the concentration of DNA. DNA absorbs strongly at A_{260} , and thus the concentration of a sample of DNA can be measured spectrophotometrically. This is only useful for determinations of purified DNA because contaminants such as RNA and protein will decrease the accuracy of the measurement. The second method is to visually compare DNA samples of known and unknown concentrations following agarose gel electrophoresis. A visual comparison will provide a rough estimate of the concentration of DNA in the unknown sample.

A. Estimation of DNA Concentration by Agarose Gel Electrophoresis

1. Prepare and pour a 0.7% agarose mini-gel as described in the previous experiment.
2. While the gel is solidifying, prepare concentration standards and plasmid DNA for electrophoresis.
 - a. Dilute 1 $\mu\text{g}/\mu\text{l}$ stock pBR322 to 100 ng, 10 ng, and 1 ng, by performing 10-fold serial dilutions in sterile water in a final volume of 10 μl . Prepare a 1- μg standard by adding 1 μl of stock pBR322 DNA to 9 μl of sterile water.
 - b. Add 2 μl of pMB1 DNA to 8 μl of sterile water.
 - c. Add 2 μl of 6X Sample Buffer to each of the five tubes and mix. Centrifuge briefly (one or two seconds) at high speed.
3. When the gel has solidified, remove the comb and load the samples. Electrophorese at 100 V for thirty minutes (or until the dye front has migrated approximately half-way down the gel).
4. Place the gel on a UV light box and compare the intensity of the pMB1 band with that of the standards. Estimate the amount of pMB1 DNA contained in the band, and determine the concentration of the pMB1 solution. For example, if the pMB1 band most closely matched the 100 ng standard, then the concentration would be 100 ng/2 μl , or 0.05 $\mu\text{g}/\mu\text{l}$.

B. Colony Transformation

1. Add 100 μl of cold CaCl_2 to two microfuge tubes, labeled "tube 1" and "tube 2." Add 100 μl of TY broth to a third tube, labeled "tube 3." Place all three tubes on ice and incubate for at least five minutes.
 2. Aseptically transfer one large, overnight colony of *E. coli* HA3 to each tube. Completely resuspend the colony so that no visible clumps remain in the tube.
 3. Return the tubes to ice and incubate for fifteen minutes.
 4. Add 100 ng of pMB1 DNA directly to the cell suspension in tubes 1 and 3. Do not add plasmid DNA to tube 2.
 5. Mix each tube gently, and incubate them on ice for thirty minutes.
 6. Remove the tubes from the ice and immediately immerse them in a 42° C waterbath for exactly ninety seconds. Following the heat shock, immediately return the tubes to ice and chill for five minutes.
 7. Add 200 μl of TY broth to each tube and gently mix. Allow the cells to recover from the heat shock by incubating the tubes in a 37° C waterbath for fifteen minutes to one hour.
 8. Using the spread-plate technique, spread onto TY-Amp agar plates:
 - a. 100 μl from tube 1 (transformation tube)
 - b. 100 μl from tube 2 (control tube)
 - c. 100 μl from tube 3 (control tube)
- Store the culture remaining in each tube at 4° C until you determine that the transformation was successful.

9. Invert the plates and incubate at 37° C overnight. After incubation, check the plates for growth. If no colonies appear on the transformation plate, centrifuge the transformation tube (tube 1) for one minute at high speed to pellet the cells. Pour off most of the supernatant, leaving a small volume in the tube. Resuspend the pellet in the remaining liquid, and spread the suspension on a TY-Amp agar plate. Incubate the plate overnight at 37° C, and examine for transformants. If no colonies appear, the transformation procedure was unsuccessful and will have to be repeated.

C. Replica Plating for Selection of Cloned Gene

1. Using the templates shown in figure 5.2, replica plate each transformant to MM/adenine and MM plates. Carefully touch one colony with the flat end of a sterile toothpick. Touch the toothpick to identical spots on the two plates, then discard it. Repeat using new sterile toothpicks until at least one hundred transformants have been replica plated. To accommodate this number of transformants, use two plates of each. Be sure to label and orient each plate properly.
2. Incubate the plates at 37° C overnight.
3. Check the plates for colonies. Cells that were transformed with pMB1 will grow both in the presence and absence of adenine.
4. Aseptically transfer one pMB1 transformant colony to a TY-Amp agar plate using the streak-plate technique. Incubate at 37° C overnight, then store the plate at 4° C for future use.

Assignment

1. Predict the possible outcomes of the subclone construction of pMB1. Is pMB1 the only recombinant molecule that may have formed?
2. Estimate the concentration of pMB1 DNA using the agarose gel estimation technique.
3. Interpret the results of the transformation and control plates.
4. Count the number of ampicillin-resistant colonies growing on the transformation plate. Determine the transformation efficiency (expressed as the number of transformants obtained per microgram of DNA).
5. After replica plating the transformants, determine which colonies represent clones of pMB1. Subculture to new media and store for future use.

Suggested Readings

- Hanahan, D. 1987. "Mechanisms of DNA Transformation," in *Escherichia coli and Salmonella typhimurium: Cellular and Molecular Biology*, Vol. II, American Society for Microbiology, Washington, D.C..
- Mandel, M., and Higa, A. 1970. "Calcium Dependent Bacteriophage DNA Infection." *Journal of Molecular Biology*, 53:159-162.
- Sambrook, J.; Fritsch, E. F.; Maniatis, T. 1989. *Molecular Cloning, A Laboratory Manual*, 2nd ed. Cold Spring Harbor Laboratory Press, NY.

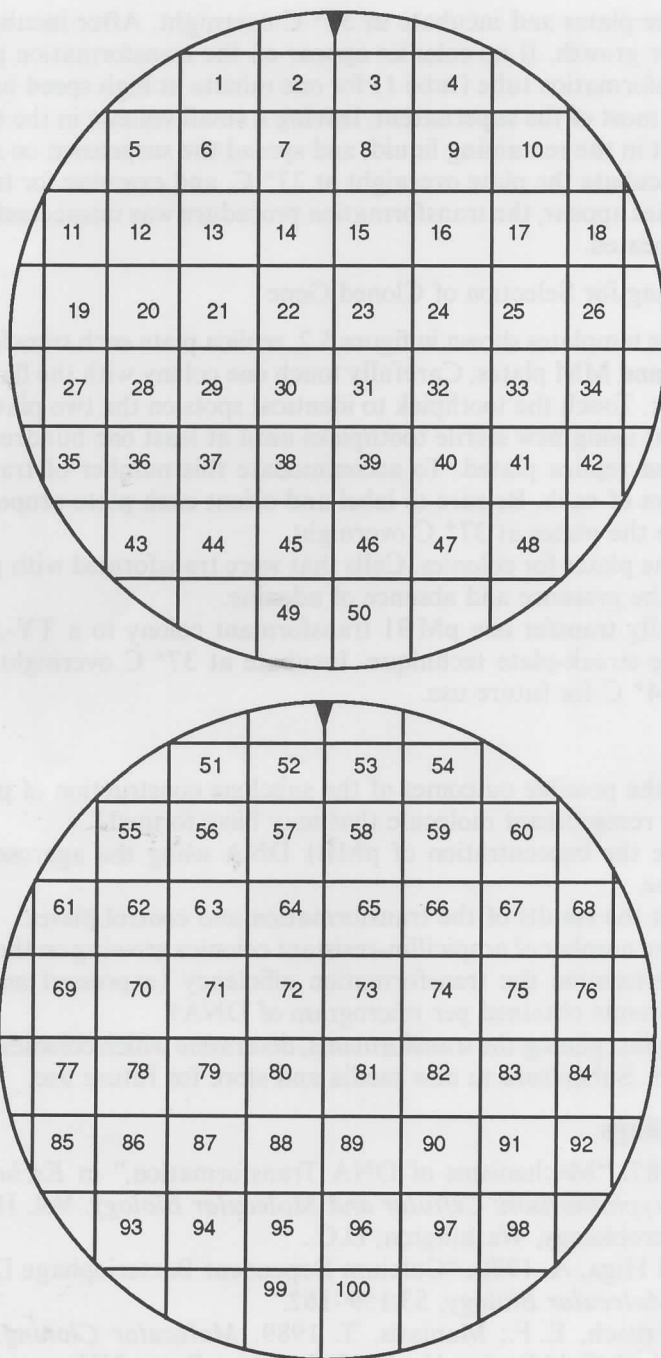


Figure 5.2. Templates for the phenotypic selection of pMB1 clones.

NOTES AND CALCULATIONS

Chapter 6

Mini-prep Isolation of Plasmid DNA

Introduction

In recent years, plasmids have become the workhorses of the molecular biologist. Their most important role is as a vector, into which foreign DNA carrying genes of interest can be ligated. The recombinant plasmid is then transformed into a host bacterium and stored for long periods. In this way, copies of important genes are saved for future use. Although the presence of a plasmid in a bacterial cell may be detected phenotypically (as we saw previously), it is often desirable to isolate the plasmid DNA from the host cell for molecular studies, such as restriction mapping and nucleotide sequencing, or for the construction of new plasmids.

Isolation of plasmid DNA from a bacterial host requires that it be separated from the cellular DNA and RNA, as well as from other cellular macromolecules such as proteins. Plasmid DNA can be isolated from host cells by first lysing the cells and then selectively precipitating out of solution the contaminating macromolecules until all that remains is the plasmid DNA. Over the years, a number of different methods have been developed to purify plasmid DNA. The most commonly used small-scale method is that of Birnboim and Doly, and is called the *alkaline lysis*, or *mini-prep*, technique.

To isolate plasmid DNA, the host cells are first treated with SDS and lysozyme in an alkaline environment. This weakens the cell walls and lyses the cells. The lysate is then neutralized with acidic sodium acetate. This treatment selectively denatures high molecular weight (chromosomal) DNA without damaging the covalently closed circular (plasmid) DNA. The presence of salt in the neutralizing buffer causes the chromosomal DNA to aggregate into an insoluble network, which precipitates out of solution. The high concentration of sodium acetate in the solution also precipitates most of the cellular RNA and protein-SDS complexes that have formed. The precipitated contaminating macromolecules are pelleted out of solution by centrifugation, leaving plasmid DNA and some residual low molecular weight RNA in solution. The remaining nucleic acids can then be precipitated from the supernatant by ethanol or isopropanol precipitation. The plasmid DNA can be completely purified from residual RNA by incubating the solution with the enzyme RNase, which selectively degrades RNA.

When this technique is performed carefully, the yield of purified plasmid DNA is quite good. The DNA obtained from a mini-prep is usually of sufficient quantity and purity that it can be cleaved with restriction enzymes, which is the goal of this experiment. pMB1 DNA will be isolated from host cells by performing mini-preps on transformed *E. coli* HA3, and the yield will be estimated by agarose gel electrophoresis. The DNA obtained from this mini-prep will be stored at 4° C for subsequent restriction map analysis.

Materials

Reagents

Solution I (50 mM glucose, 25 mM Tris pH 8.0, 10 mM EDTA, with 4 mg/ml lysozyme added immediately before use); Solution II (0.2 N NaOH, 1% SDS, freshly made); Solution III (potassium acetate, pH 4.8); phenol; Sevag; TE pH 8.0; 1 µg/µl pBR322 DNA; 50X TAE; agarose; 10 mg/ml ethidium bromide; 6× Sample Buffer; sterile distilled water

Supplies and Equipment

Microfuge tubes; microcentrifuge; ice; micropipettor and sterile tips; mini-gel apparatus; power supply

Cultures

TY-Amp plate culture of *E. coli* HA3/pMB1 transformants

Procedure

Twenty-four hours before the scheduled lab period, subculture one isolated colony from your *E. coli* HA3/pMB1 transformation plate to 3 ml of TY-Amp broth. Incubate overnight at 37° C.

A. Agarose Mini-gel Preparation

1. Prior to or during the mini-prep procedure, prepare a 0.7% agarose mini-gel and 250 ml of running buffer (1X TAE).

B. Mini-prep Procedure

1. Fill two microfuge tubes nearly full with overnight *E. coli*/pMB1 culture (approximately 1.5 ml per tube).
2. Centrifuge the tubes for one minute at high speed in a microcentrifuge.
3. Without disrupting the pellets, remove as much of the media as possible with a Pasteur pipette.
4. Add 100 µl of ice cold Solution I to the pellets. Vortex to completely resuspend.
5. Incubate the tubes at room temperature for exactly five minutes.
6. Add 200 µl of freshly made, room-temperature Solution II. Close the caps and mix vigorously by rapidly inverting the tubes several times.
7. Incubate the tubes on ice for exactly five minutes.
8. Add 150 µl of ice cold Solution III. Close the caps and gently mix by slowly inverting the tubes ten times.

9. Incubate the tubes on ice for exactly five minutes. The solution will begin to take on a milky appearance as proteins and high molecular weight DNA precipitate out of solution.
10. Centrifuge the tubes for five minutes on high speed in a 4° C microcentrifuge to pellet the precipitate to the bottom of the tubes.
11. Using a micropipettor set at 400 μ l, carefully remove the supernatant from each tube and transfer it to new microfuge tubes. Discard the tubes containing the protein-SDS-chromosomal DNA precipitate.
12. Add 400 μ l of a 1:1 solution of phenol/Sevag to each tube and mix by vortexing. Spin on high speed for one minute. Remove the upper aqueous phase and transfer to two new tubes.
13. Measure the volume of the DNA solutions. Add two volumes of room-temperature 95% ethanol to each tube, and mix by vortexing.
14. Incubate the tubes at room temperature for two minutes.
15. Spin for five minutes in a room-temperature microcentrifuge at high speed.
16. Decant the supernatant, and invert the tubes over a paper towel to drain off the ethanol.
17. Wash the pellets with 1 ml of 70% ethanol. Flick the tubes with your finger several times, then recentrifuge.
18. Decant the supernatant and invert the tubes over a paper towel to drain off the majority of the ethanol.
19. Evaporate any remaining ethanol by directing a stream of hot air from a blow dryer over the tops of the open tubes.
20. Add 25 μ l of TE buffer (pH 8.0) to both tubes, and allow them to stand at room temperature for five minutes. Resuspend the DNA by gently pipetting up and down with a micropipettor. When both pellets are completely resuspended, combine the solutions into one tube.

C. Estimation of DNA Concentration by Agarose Gel Electrophoresis

1. Prepare DNA concentration standards using 1 μ g/ μ l pBR322 DNA, to 1 μ g, 100 ng, 10 ng and 1 ng, as described in the previous experiment.
2. Add 2 μ l of mini-prep DNA to 8 μ l of TE buffer in a fifth microfuge tube.
3. Add 2 μ l of 6X Sample Buffer to each tube and mix. Centrifuge the tubes for two seconds in a microfuge.
4. Load the standards and mini-prep DNA into sample wells in a 0.7% agarose mini-gel. Add running buffer to the buffer chambers, and electrophorese at 100 V until appropriate migration has occurred (approximately thirty minutes).
5. Place the gel on a UV light box and compare the intensity of the mini-prep DNA band with that of the standards. Estimate the amount of DNA in the band, and determine the concentration of the DNA sample.
6. Label the mini-prep DNA pMB1 and include the concentration on the tube. Store the DNA at either 4° C or at -20° C.

Assignment

1. Describe the functions of Solutions I, II, and III in the mini-prep procedure.
2. Estimate the concentration of mini-prep DNA from the results of the agarose gel electrophoresis. Determine the total quantity (in μg) of pMB1 DNA obtained from the mini-prep.

Suggested Readings

- Birnboim, H. C., and Doly, J. 1979. "A Rapid Alkaline Extraction Procedure for Screening Recombinant Plasmid DNA." *Nucleic Acids Research*, 7:1513-1523.
- Sambrook, J.; Fritsch, E. F.; Maniatis, T. 1989. *Molecular Cloning: A Laboratory Manual*, 2nd ed. Cold Spring Harbor Laboratory Press, NY.

NOTES AND CALCULATIONS

Chapter 7

Restriction Mapping of Plasmid DNA

Introduction

One way in which scientists can determine the success or failure of plasmid transformation experiments is to reisolate the plasmid DNA from the bacterial host, as illustrated in the previous experiment. Isolation of a plasmid from a transformed bacterium, however, does not guarantee that the gene of interest has been cloned. Conventionally, a cloned gene is often defined by its location relative to restriction sites on a plasmid. Therefore, one of the first steps in characterizing a recombinant molecule is to create a restriction map. The information obtained from the restriction map, in conjunction with the data obtained from genetic selection experiments, provides researchers with a comprehensive picture of the recombinant plasmid.

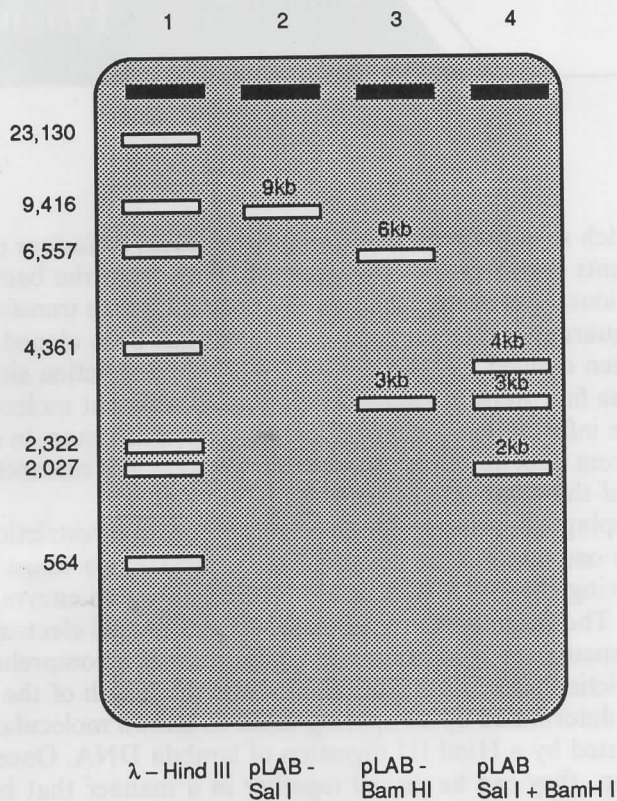
Restriction mapping analysis provides a means of locating restriction sites and their positions relative to one another on plasmid DNA. Restriction maps of plasmids are constructed by cleaving the DNA with one or more restriction enzymes to generate a series of fragments. The fragments are subjected to agarose gel electrophoresis and are separated. The fragments are then oriented so as to provide a comprehensive picture of the location of restriction sites. To do this accurately, the length of the restriction fragments must first be determined by comparing them to known molecular size standards, such as those generated by a Hind III digestion of lambda DNA. Once the sizes of the fragments are known, they can be pieced together in a manner that is consistent with the available restriction fragment data.

As previously described, restriction digestion reactions must be carried out under strictly controlled conditions. One drawback to performing digestions with more than one enzyme is in meeting the optimal conditions for each type of enzyme. For example, a double digestion with BamH I and Sal I could not be performed in the same sodium chloride reaction buffer because Sal I has a higher salt requirement. In order to perform a double digestion with these two enzymes, the DNA would have to be digested first with EcoR I at low salt, and then the salt concentration would have to be increased before adding Sal I to the reaction. This effectively doubles the time needed to perform a restriction digestion.

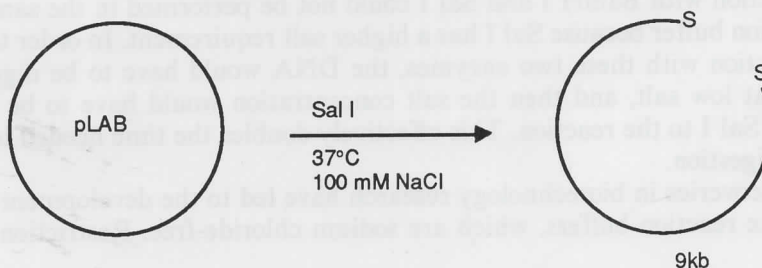
Recent discoveries in biotechnology research have led to the development of potassium glutamate reaction buffers, which are sodium chloride-free. Restriction enzymes

work over a much broader concentration range in potassium glutamate buffers. Therefore, double digestions such as the one described above, which use to require elaborate manipulations, can be done simultaneously in the same reaction buffer.

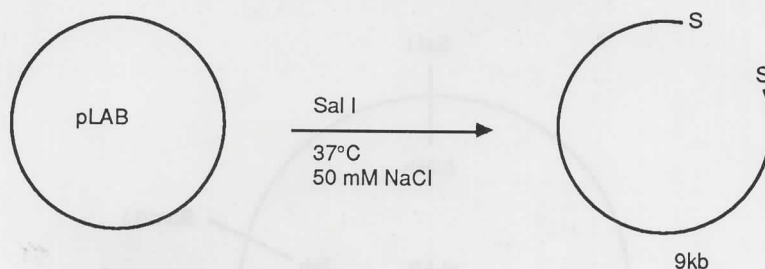
Constructing restriction maps is an intuitive process. For example, consider the hypothetical plasmid pLAB. Restriction digestions of pLAB with the enzymes Sal I and BamH I, and a double digestion with both enzymes generates the following fragments:



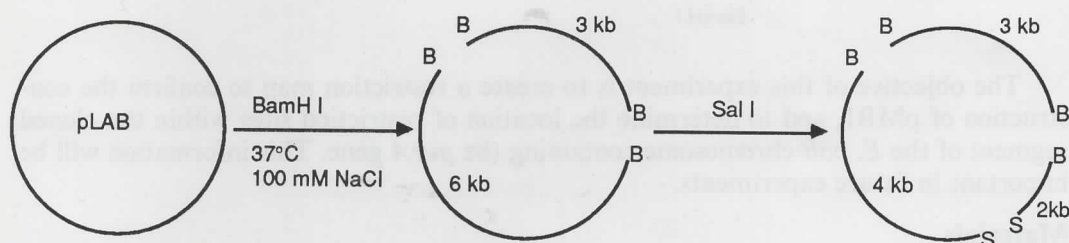
The enzyme Sal I cuts the plasmid at a unique site to yield a single 9 kb band. This represents the full linear length of the plasmid, as shown below:



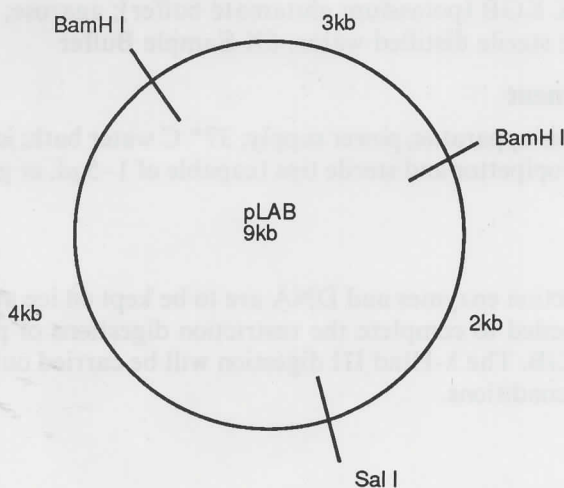
BamH I cuts the plasmid twice to generate two fragments, one 6 kb long, and the other 3 kb long.



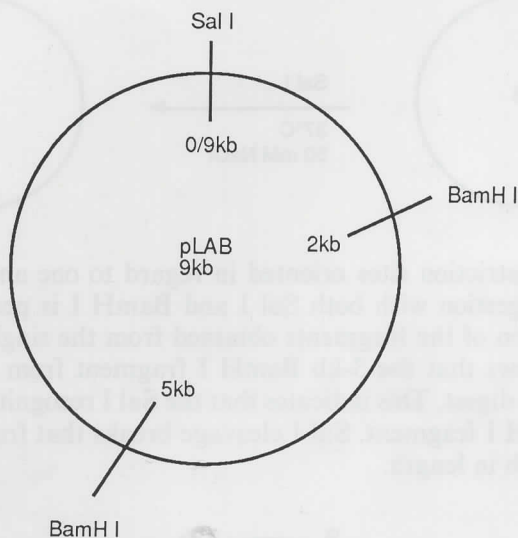
How are these restriction sites oriented in regard to one another? To answer this question, a double digest with both Sal I and BamH I is performed to yield three fragments. Comparison of the fragments obtained from the single digest to those from the double digest shows that the 3-kb BamH I fragment from the single digest is retained in the double digest. This indicates that the Sal I recognition site lies somewhere within the 6-kb BamH I fragment. Sal I cleavage breaks that fragment into two pieces that are 4 kb and 2 kb in length.



Therefore, the restriction map of pLAB could be written as follows:



By convention, the unique restriction site is usually placed at the top of the map and is called site 0. Other restriction sites are then placed on the map in a clockwise manner. In our example then, the restriction map could be written:



The objective of this experiment is to create a restriction map to confirm the construction of pMB1, and to determine the location of restriction sites within the cloned segment of the *E. coli* chromosome containing the *purA* gene. This information will be important in future experiments.

Materials

Reagents

Mini-prep pMB1 DNA; stock λ DNA; Hind III and 10X reaction buffer (low salt); Sal I; Nde I; EcoR I; 10X KGB (potassium glutamate buffer); agarose; 50X TAE; 10 mg/ml ethidium bromide; sterile distilled water; 6X Sample Buffer

Supplies and Equipment

Mini-gel electrophoresis apparatus; power supply; 37° C water bath; ice; microfuge tubes; microcentrifuge; micropipettor and sterile tips (capable of 1–5 μ l, or glass microcapillary pipettes)

Procedure

Remember, restriction enzymes and DNA are to be kept on ice at all times. In order to reduce the time needed to complete the restriction digestions of pMB1, they will be performed in 1.5X KGB. The λ -Hind III digestion will be carried out under normal (50 mM NaCl) reaction conditions.

A. Restriction Digestion of pMB1

1. Prepare experimental protocols for the digestions shown below:

Tube	dH ₂ O	DNA	10X Buffer	Enzyme	Volume Total
1	_____	5 μ g λ	2 μ l	5 U Hind III	20 μ l
2	_____	1 μ g pMB1	3 μ l	2 U Sal I	20 μ l
3	_____	1 μ g pMB1	3 μ l	2 U Nde I	20 μ l
4	_____	1 μ g pMB1	3 μ l	2 U EcoR I	20 μ l
5	_____	1 μ g pMB1	3 μ l	2 U Sal I, Nde I	20 μ l
6	_____	1 μ g pMB1	3 μ l	2 U Sal I, EcoR I	20 μ l
7	_____	1 μ g pMB1	3 μ l	2 U Nde I, EcoR I	20 μ l

2. Add the reactants to labeled microfuge tubes, and thoroughly mix the contents by flicking the tubes with your fingers. Spin the tubes briefly in a microfuge.
3. Incubate the tubes in a 37° C waterbath for at least one hour.

B. Agarose Gel Electrophoresis

1. Pour a 0.7% agarose mini-gel and prepare 250 ml of running buffer (1X TAE).
2. When the gel has solidified, remove the comb and pour running buffer into the buffer chambers of the electrophoresis apparatus.
3. When the digestions have incubated for an appropriate amount of time, add 4 μ l of 6X Sample Buffer to each tube and briefly spin in a microfuge. Load the digestions into the sample wells.
4. Electrophorese at 50 volts until appropriate migration has been achieved (one to two hours). Lower voltage is used to optimize separation of the fragments.
5. Place the gel on a UV light box and examine the fragments. If they are well separated, take a photograph of the gel. It may be necessary to adjust the exposure time in order to obtain a clear picture. If the bands do not appear to be completely separated, place the gel back into the electrophoresis apparatus and run for one-half to one hour longer.

C. Construction of a Restriction Map

1. Using the λ -Hind III digestion as molecular weight standards, prepare a standard curve as previously described.
2. Determine the sizes of *all* of the fragments generated in each digestion.
3. Construct a restriction map of pMB1 that fits the restriction data.

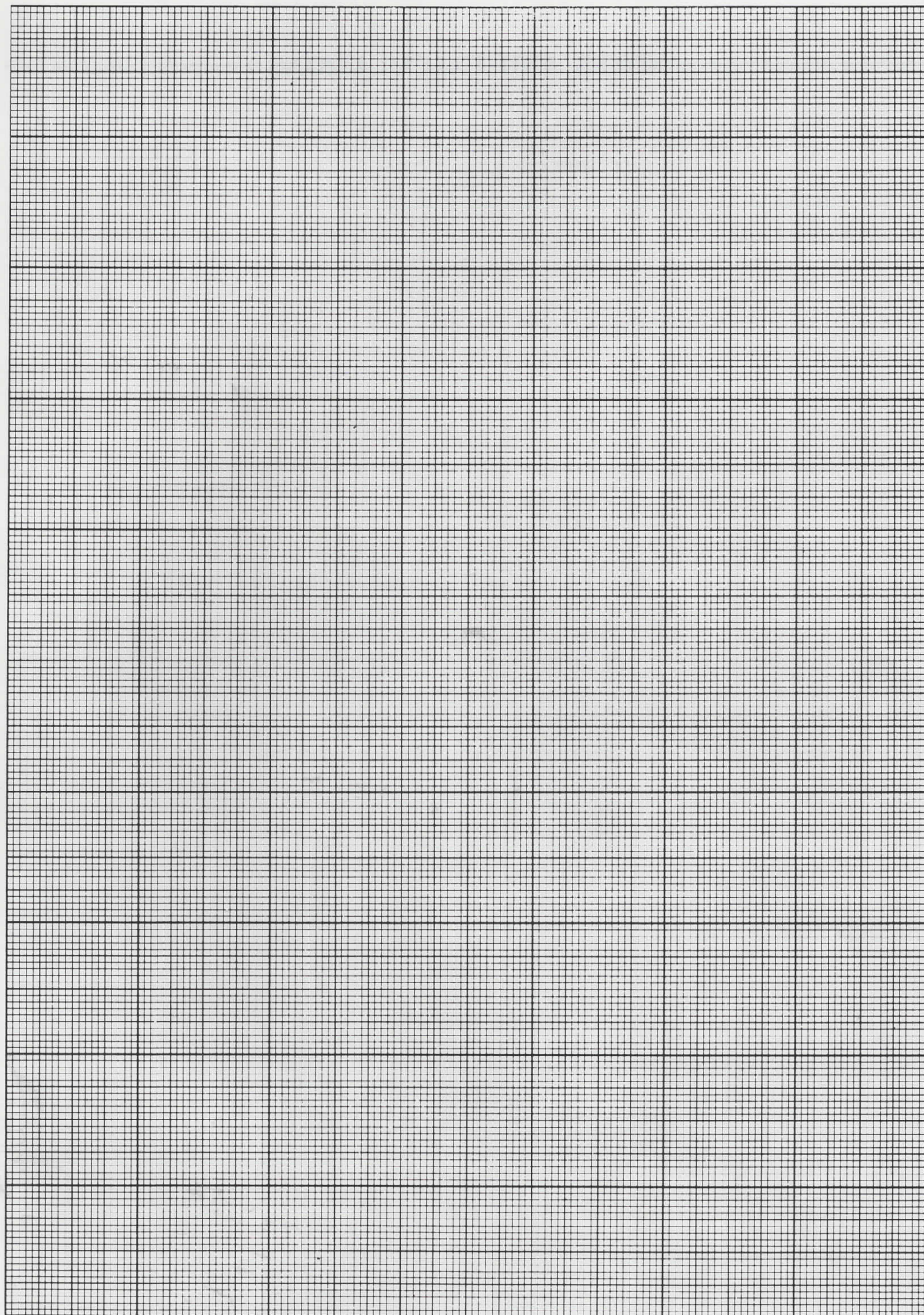
Assignment

1. Prepare experimental protocols for each restriction digestion.
2. Create a table that shows the distance each restriction fragment migrated (in cm), 1/d, and the size of the fragments for each single and double digestion.
3. Draw two restriction maps of pMB1; one showing just the Sal I and Nde I sites, and the other showing the Sal I, Nde I and EcoR I sites.

4. Compare the restriction map showing the Sal I and Nde I sites to the expected outcome of the subcloning experiment. By combining the results of the genetic selection experiment and the restriction map of pMB1, determine if the construction of plasmid pMB1 was successful.

Suggested Readings

- Rodriguez, R. L., and Tait, R. C. 1983. *Recombinant DNA Techniques: An Introduction*. Addison-Wesley Pub. Co., Massachusetts.
- Watson, J. D.; Tooze, J.; and Kurtz, D. T. 1983. *Recombinant DNA: A Short Course*. Scientific American Books, W. H. Freeman and Co., New York.



NOTES AND CALCULATIONS

Chapter 8

In vitro Mutagenesis of a Cloned Gene: Kan Cartridge Mutagenesis

Introduction

One way to facilitate chromosomal mapping is to create mutations within particular genes. Inactivation of a gene by mutagenesis techniques allows scientists to make genetic maps by relating the loss of gene function to a location on the chromosome. Analysis of mutations within a particular gene has also become important to medical research because many diseases, both inherited and acquired, trace their origins to chromosomal mutations. Once a gene has been mutagenized, it can be isolated by genetic selection. However, if the phenotype for the mutant is not easily selectable, isolation and characterization become more difficult. Therefore, mutagenesis techniques often involve the use of a selectable marker, such as antibiotic resistance. A gene tagged with a marker can be easily selected from a mutagenized pool of clones, even if the phenotype associated with the gene is unknown.

Mutations in a gene of interest can be obtained by transposon mutagenesis. *Transposons*, or *transposable elements*, are small pieces of double-stranded DNA that move about freely and integrate into random sites in chromosomal DNA. Many transposons carry antibiotic resistance genes. Insertion of a transposon results in insertional inactivation of affected genes. Bacterial cells containing a mutation of interest can then be isolated by subjecting a pool of mutagenized cells to genetic selection and screening for cells that are antibiotic resistant and auxotrophic for the desired trait. Transposon mutagenesis works best on chromosomal genes because plasmids are poor hosts for transposons. Once the chromosomal mutant has been isolated, the mutation can be “swapped” onto a plasmid by recombination between homologous regions on the plasmid and chromosomal DNA. The mutagenized gene can then be fully characterized by restriction map analysis (see figure 8.1).

A disadvantage to using transposons for mutagenesis experiments is that insertional activation of the gene often inactivates downstream genes within the same transcriptional unit (a *polar effect*). Also, insertion of a transposon into a gene disrupts and inactivates a gene, as opposed to making small-scale changes in the DNA. Single base changes in genes have become especially significant to genetic studies because they occur naturally at a relatively high rate. Chemically induced mutations result in specific or

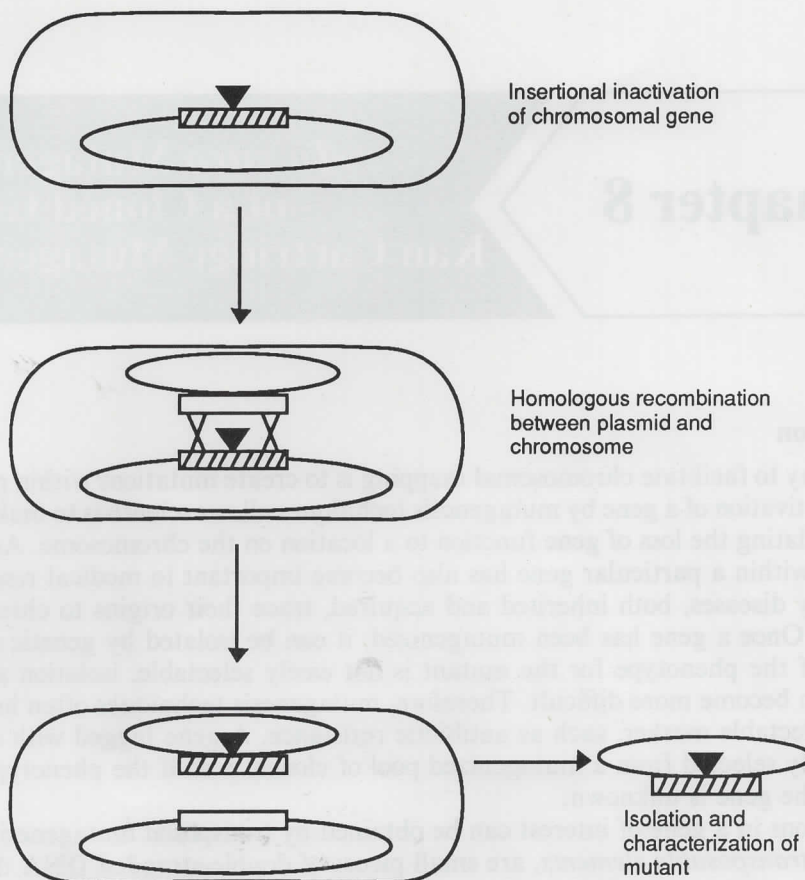


Figure 8.1. Transposon mutagenesis of a cloned gene.

random single base changes in a sequence of DNA, thus eliminating the polar effect (see figure 8.2). Mutant proteins can be expressed from genes altered by chemical mutagenesis and can be studied biochemically to determine the effect of the mutation on expression of the affected genes and related genetic systems. Chemical mutagenesis can be performed on chromosomal or cloned DNA, but must be carried out under strictly controlled conditions or the DNA can become drastically altered. Also, the chemicals used in mutagenesis experiments are usually carcinogenic. Therefore, extreme caution is required during chemical mutagenesis experiments.

Mutations can also be created in genes following insertion of a defined fragment of DNA carrying an antibiotic resistance marker into a restriction site within the sequence of a cloned gene. This results in insertional inactivation of the cloned gene, and tags the mutation with a selectable marker. A 1.5-kb *EcoR* I fragment from the plasmid pUC-4K, as shown in figure 8.3, is an excellent cartridge to use in mutation studies. This

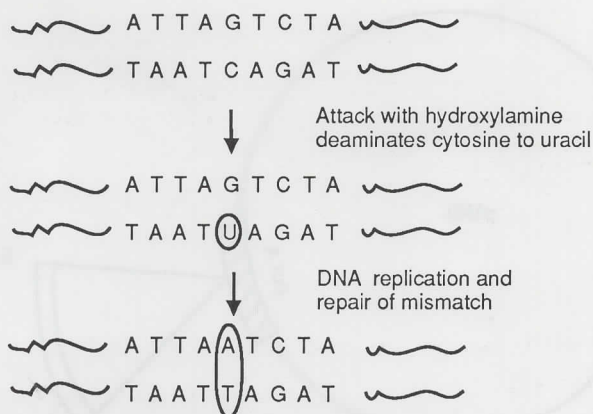


Figure 8.2. Chemical mutagenesis can result in the formation of single base-pair changes in a sequence of DNA.

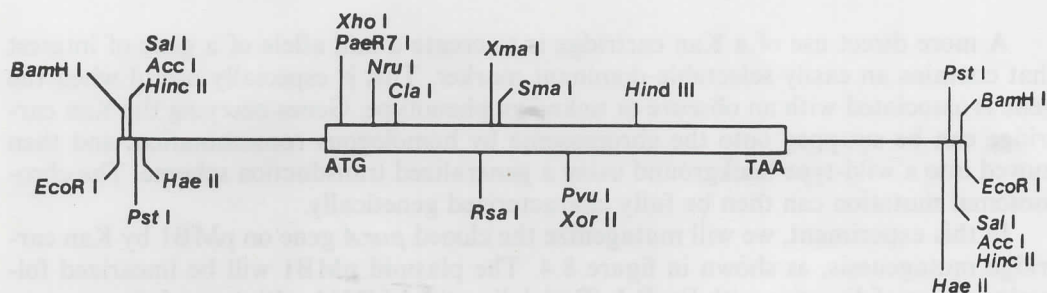


Figure 8.3. A Kan cartridge (the Kan® GenBlock from Pharmacia). From *Pharmacia 1988 Product Catalog*. Copyright © Pharmacia Inc., Piscataway, NJ.

cartridge (*Kan cartridge*) has Sal I, Pst I, BamH I, and Acc I recognition sites at each end, so it can be inserted into any of these restriction sites within a gene of interest. It also carries the APH gene, which encodes the enzyme aminoglycoside 3'-phosphotransferase, for kanamycin resistance.

The Kan cartridge was originally developed to create codon insertions in cloned genes. The cloned gene was first cut with one of the restriction enzymes with a site at either end of the cartridge. The cartridge was then ligated into the restriction site, rendering kanamycin resistance to transformants carrying the mutagenized plasmid. The middle portion of the cartridge, including the APH gene, could be removed from the plasmid by cutting with a second restriction enzyme, leaving insertions of one to several codons in the gene of interest. These types of mutations can be created in-frame in an expression system to yield quantities of purified mutant protein for biochemical studies.

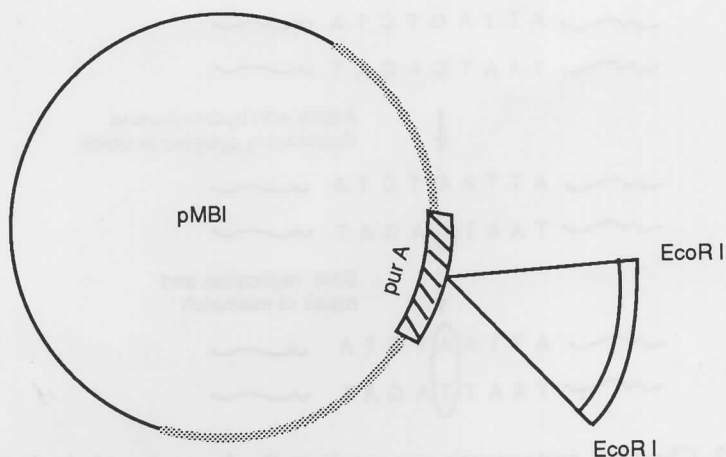


Figure 8.4. Kan cartridge mutagenesis of the cloned *purA* gene on pMB1.

A more direct use of a Kan cartridge is to create a null allele of a gene of interest that contains an easily selectable dominant marker. This is especially useful when the gene is associated with an obscure or unknown phenotype. Genes carrying the Kan cartridge can be swapped onto the chromosome by homologous recombination, and then moved into a wild-type background using a generalized transduction scheme. The chromosomal mutation can then be fully characterized genetically.

In this experiment, we will mutagenize the cloned *purA* gene on pMB1 by Kan cartridge mutagenesis, as shown in figure 8.4. The plasmid pMB1 will be linearized following a partial digestion with EcoR I. Partial digestion of DNA with a restriction enzyme simply means that the DNA is not digested to completion. For example, the partial digestion of the plasmid pLAB with BamH I would yield the products shown in figure 8.5.

To mutagenize the gene, the cartridge will be ligated into the EcoR I site within the gene and transformed into an *E. coli* adenine auxotroph. Clones containing the mutagenized gene will be isolated by genetic selection, and then stored for later use.

Materials

Media

TY-Kan agar plate, TY-Amp agar plates, MM plates, MM/adenine plates

Reagents

Purified pUC-4K DNA, or Kan^R GenBlock (Kan cartridge from Pharmacia); pMB1 DNA; λ DNA; pBR322 DNA; 250 mM EDTA; phenol; Sevag; 95% ethanol; 70% ethanol; Hind III and 10X buffer; EcoR I and 10X buffer; T4 DNA Ligase and 10X buffer; agarose; sterile water; 50X TAE; 10 mg/ml ethidium bromide; 6X Sample Buffer; 100 mM CaCl₂; TE pH 8.0.

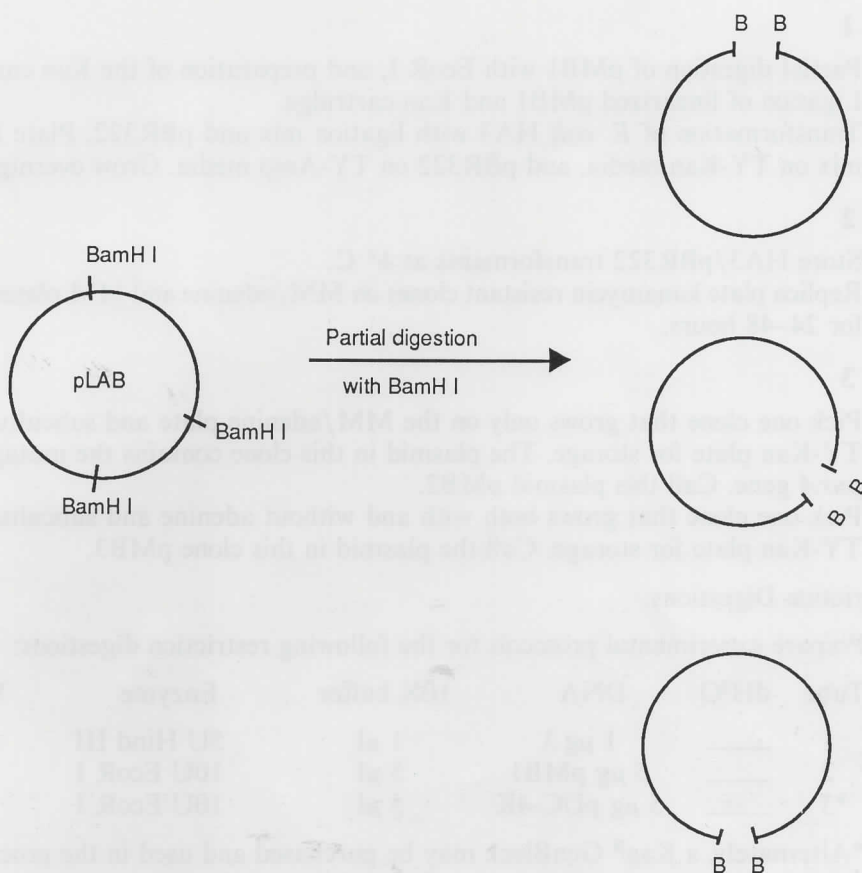


Figure 8.5. Plasmid pLAB partial digestion with BamH I.

Equipment and Supplies

Mini-gel electrophoresis apparatus; power supply; microcentrifuge; microcentrifuge tubes; micropipettors and sterile tips; 37° C waterbath; 12° C waterbath; UV light box; turntable; glass spreader; sterile toothpicks

Cultures

TY (adenine) plate culture of *E. coli* HA3 grown at 37° C overnight.

Procedure

This experiment requires more than one day to complete. It will be necessary for students to return to the laboratory to complete the procedure. Note that only the first day involves a lengthy lab period. The following days require only a short time in the lab. A suggested schedule is given below:

Day 1

1. Partial digestion of pMB1 with EcoR I, and preparation of the Kan cartridge.
2. Ligation of linearized pMB1 and Kan cartridge.
3. Transformation of *E. coli* HA3 with ligation mix and pBR322. Plate ligation mix on TY-Kan media, and pBR322 on TY-Amp media. Grow overnight.

Day 2

4. Store HA3/pBR322 transformants at 4° C.
5. Replica plate kanamycin resistant clones on MM/adenine and MM plates. Grow for 24–48 hours.

Day 3

6. Pick one clone that grows only on the MM/adenine plate and subculture to a TY-Kan plate for storage. The plasmid in this clone contains the mutagenized *purA* gene. Call this plasmid pMB2.
7. Pick one clone that grows both with and without adenine and subculture to a TY-Kan plate for storage. Call the plasmid in this clone pMB3.

A. Restriction Digestions

1. Prepare experimental protocols for the following restriction digestions:

Tube	dH ₂ O	DNA	10X buffer	Enzyme	Volume
1	—	1 µg λ	1 µl	5U Hind III	10 µl
2	—	5 µg pMB1	5 µl	10U EcoR I	50 µl
*3	—	5 µg pUC-4K	5 µl	10U EcoR I	50 µl

*Alternately, a Kan^R GenBlock may be purchased and used in the procedure.

2. Add the reactants to labeled microfuge tubes and place them in a 37° C waterbath. Incubate tubes 1 and 3 for one hour.
3. Remove 10 µl aliquots from tube 2 at the following time intervals: 5, 10, 15, 30 and 60 minutes. Place each aliquot in a labeled microfuge tube, and add 1 µl of 250 mM EDTA to stop the reaction. Hold the tubes on ice until all of the restriction digestions are complete.

B. Agarose Gel Electrophoresis

1. While the restriction reactions are incubating, prepare a 0.7% agarose mini-gel and 250 ml of running buffer (1X TAE).
2. When the digestions are complete, add 2 µl of 6X Sample Buffer to each tube and mix. Spin briefly in a microfuge.
3. Load the digestions onto the agarose gel and electrophorese at 75 V until appropriate migration has occurred (1/2 to 1 hour).
4. Place the gel on a UV light box to determine the extent of migration. Look especially at the lanes holding the five time points of the pMB1–Eco RI digestion. You are only interested in the lane containing the best partial digestion pMB1, which will have a band representing the full-length linear plasmid cut once with EcoR I. Take a photograph of the gel.

5. Using the λ -Hind III standards as a guide, slice the 1.5-kb fragment from the pUK-EcoR I digestion and the full-length linear pMB1-EcoR I band from the gel. Place both gel slices into a single microfuge tube.
6. Extract the DNA from the gel slices using the procedure described in chapter 4.
4. Ethanol precipitate the DNA and then resuspend in 10 μ l TE.

C. Ligation Reaction

1. To ligate the Kan cartridge into pMB1, set up the following ligation reaction in a sterile microfuge tube:

3 μ l sterile dH₂O
 2 μ l 10X Ligation buffer
 10 μ l DNA
 5 μ l T4 DNA Ligase

2. Incubate the tube for 30 minutes at 12° C. The ligation can be incubated overnight if time is running short, and the procedure can be continued on the following day.

D. Transformation

1. Using the colony transformation procedure described in chapter 5, transform the following plasmids into *E. coli* HA3:

- a. ligation products—10 μ l
- b. pBR322—0.1 μ g of purified DNA

Store the remaining ligation mix at 4° C. If laboratory time is running short, the cells can be incubated in TY broth overnight at 37° C following the heat shock and can be plated on antibiotic media the following day.

2. Plate the transformed cells on antibiotic media:
 - a. HA3/ligation product: plate 100 μ l on a TY-Kan plate.
 - b. HA3/pBR322: plate 100 μ l on a TY-Amp plate.
 - c. Plate 100 μ l of HA3 without a plasmid on a TY-Amp and a TY-Kan plate. (Control).

Incubate the plates at 37° C overnight. Store the remainder of the cultures at 4° C until it is determined that the transformations were successful.

E. Replica Plating to Select for Mutagenized Gene

1. Store the HA3/pBR322 transformation plate at 4° C for future use.
2. Replica plate 100 of the kanamycin resistant colonies onto MM and MM/adrenaline plates, as described in chapter 5.
3. Incubate the plates at 37° C for 24 to 48 hours, until good-sized colonies have grown.
4. Subculture one of the *purA*⁻ clones to a TY-Kan plate for storage. Label this plate "HA3/pMB2," to indicate that the cells contain a plasmid with the Kan cartridge insert in the *purA* gene.
5. Subculture a *purA*⁺ clone to a TY-Kan plate for storage. Label this plate "HA3/pMB3" to indicate that the Kan cartridge insert is in a site other than the *purA* gene.

6. Incubate the plates at 37° C overnight. Store the plates at 4° C for use in the following experiment.

Assignment

1. Prepare experimental protocols for the restriction digestions performed in this lab.
2. Indicate on the photograph the fragments you used in the construction.
3. Why was it necessary to perform a partial digestion of pMB1 with EcoR I? Draw all of the possible restriction products generated in the reaction.
4. Draw all of the possible outcomes of ligation of the Kan cartridge into the linearized form of pMB1.
5. Determine the transformation efficiency of the ligation products into HA3.

Suggested Readings

Rodriguez, R. L., and Tait, R. C. 1983. *Recombinant DNA Techniques: An Introduction*. Addison-Wesley Pub. Co., Massachusetts.

Sambrook, J.; Fritsch, E. F.; Maniatis, T. 1989. *Molecular Cloning: A Laboratory Manual, 2nd ed.* Cold Spring Harbor Laboratory Press, NY.

NOTES AND CALCULATIONS

Chapter 9

Maxi-cell Analysis of a Cloned and Mutagenized Gene

Introduction

Restriction mapping provides scientists with an excellent means of characterizing a recombinant plasmid. In many cases, however, it becomes necessary to confirm further the genetic function of the cloned DNA. This can be accomplished by looking at the gene's protein product. Three of the most popular methods used to study the protein products of cloned or otherwise manipulated DNA are *in vitro* transcription-translation systems, mini-cell analysis, and maxi-cell analysis.

The expression of genetic information from cloned genes first involves the transcription of the DNA into an intermediary molecule, *messenger RNA (mRNA)*. The mRNA molecules are translated into a colinear sequence of amino acids, the primary structure of the synthesized protein. *In vitro* translation systems involve the use of cell extracts that contain only the components required to translate an mRNA molecule into protein. *In vitro* systems are usually cell-free, which allows protein biosynthesis to progress without interference from other cellular systems. The mRNA can be directly purified from a cell or tissue, or can be made from a gene in an *in vitro* transcription system. These types of systems have been useful in the study of proteins that are difficult to purify. If the gene can be cloned or the transcriptional product isolated, then the protein product can be synthesized and studied (See figure 9.1).

The mini-cell technique involves the use of mutant *E. coli* cells that produce small, nonviable cells following cell division. These small cells are called *mini-cells*, and are shown in figure 9.2. Mini-cells do not receive chromosomal DNA following cell division, but they do obtain plasmids from the parent cell. Mini-cells are capable of transcription and protein synthesis for a short time following isolation, and can therefore be used as a system for plasmid-coded protein biosynthesis.

The maxi-cell system also involves the use of a mutant *E. coli* strain. These cells are *uvrA* mutants. The UvrA protein is an enzyme that repairs DNA damage occurring after exposure to ultraviolet light. Irradiation of this strain results in severe chromosomal damage, thus inhibiting chromosomally directed protein synthesis and cell growth. The irradiated cells are called *maxi-cells* (to distinguish them from mini-cells). Maxi-cells continue to synthesize plasmid-coded proteins because all of the necessary cellular machinery remains present in the cells. The gene products can then be assayed using autoradiography following SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel

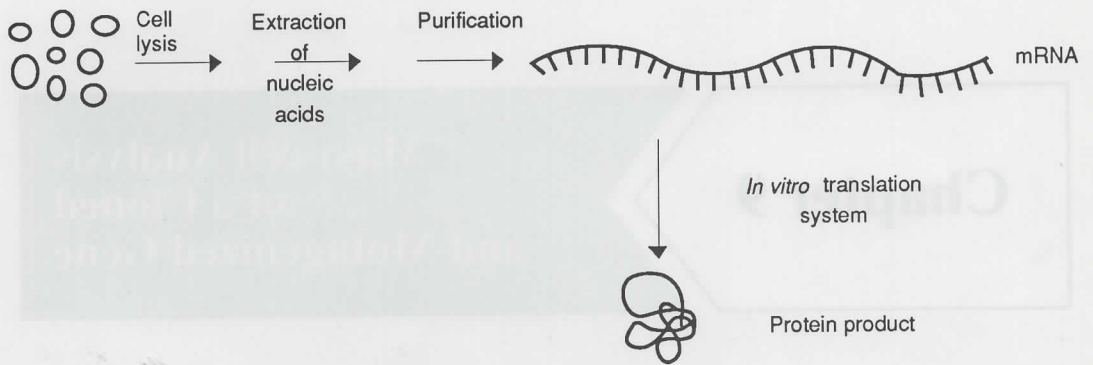


Figure 9.1. *In vitro* translation of a protein product from RNA isolated from cells.

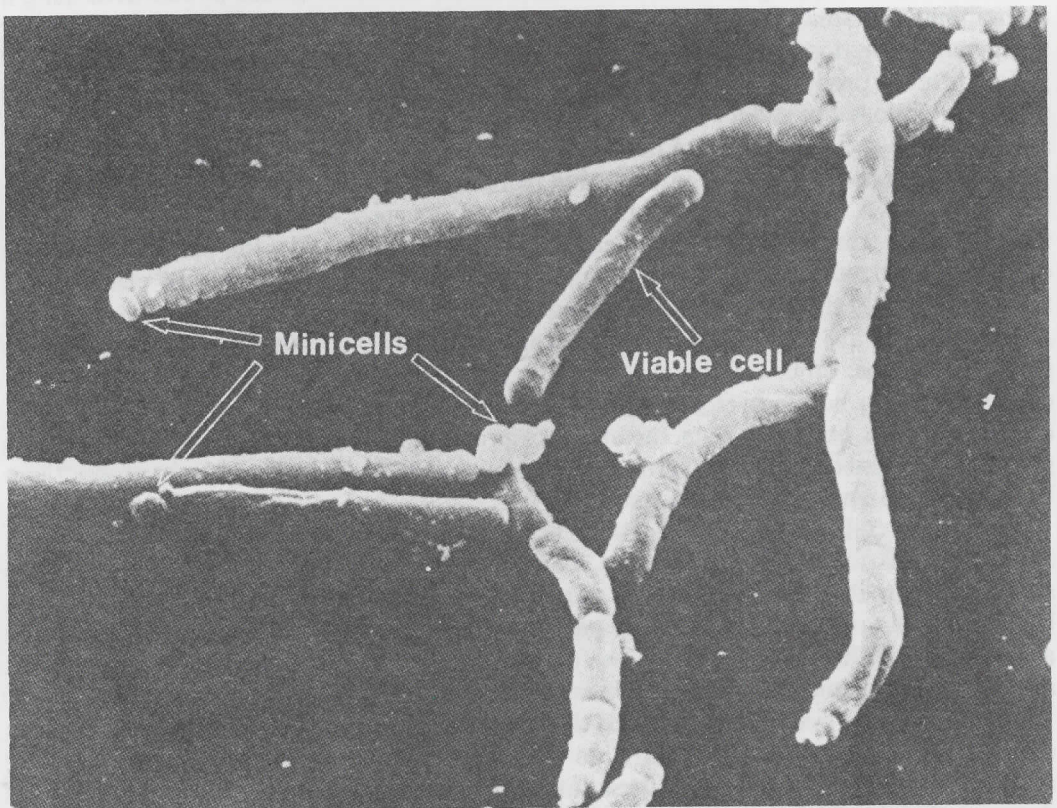


Figure 9.2. Mini-cells as seen by electron microscopy. Ullrich et al, *SCIENCE* 196: 1313, 1977; Courtesy of J. Shine and A. Ullrich.

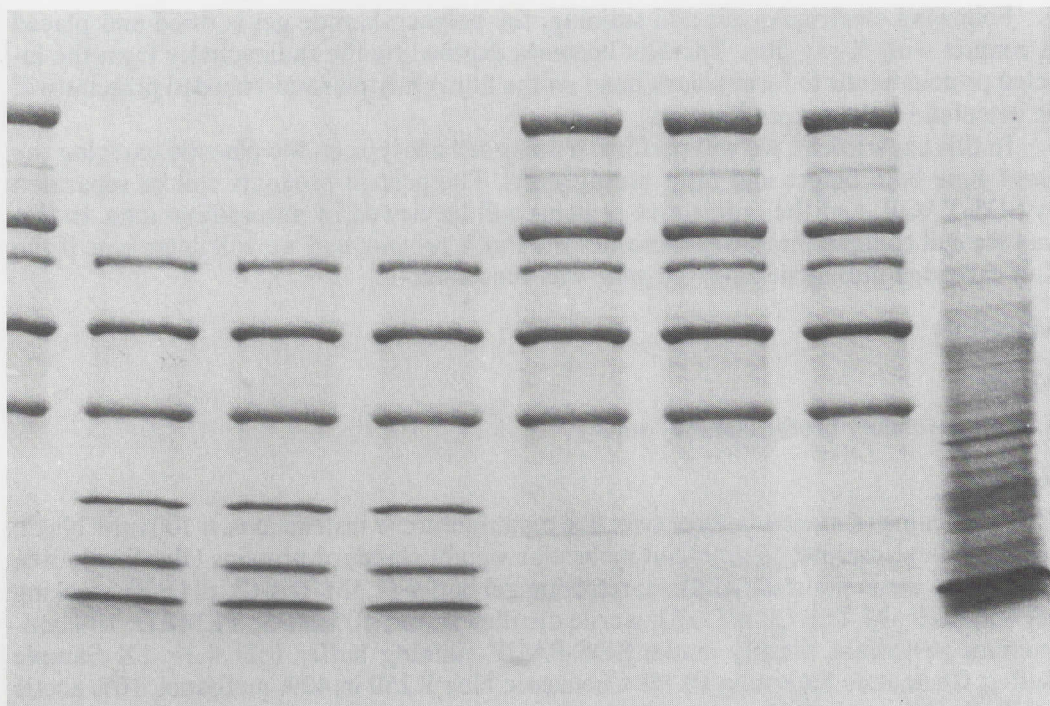


Figure 9.3. An SDS-PAGE gel showing Coomassie blue-stained protein bands.
Courtesy of Bio-Rad Laboratories.

electrophoresis). The amino acid methionine, carrying a radioactive sulfur tag, is added to the system. As protein synthesis proceeds, the labeled methionine becomes incorporated into the growing polypeptide chain along with unlabeled amino acids. This forms a radioactive protein product that was synthesized from the genes on the plasmid. These protein products are then separated from one another on a polyacrylamide gel.

The protein samples to be studied are first boiled in an extraction buffer containing SDS (an anionic detergent) and β -mercaptoethanol (a reducing agent). The proteins are denatured, and the SDS imparts a uniform negative charge to the denatured molecule. The negative charges covering the protein repel each other; thus, the protein takes on a linear conformation. When an electric field is applied to the gel, the proteins will migrate through the gel toward the anode. The polyacrylamide gel through which they travel is a matrix of cross-linked acrylamide and bis-acrylamide monomers. This matrix has the effect of sieving the linear proteins on the basis of size, with smaller proteins migrating more quickly and thus farthest through the gel. Visualization of the proteins is accomplished by staining the gel with Coomassie blue (figure 9.3), a protein dye that produces blue bands in the gel where the protein stopped migrating. Molecular weight standards (proteins of known molecular weight) are run on the gel along with proteins of unknown weight. By comparing the bands representing the standard proteins with the bands representing the unknowns, a molecular weight estimation can be made for the unknown proteins.

Following electrophoresis and staining, the polyacrylamide gel is dried and placed in contact with X-ray film. The film becomes exposed by the radioactivity from the labeled protein bands to form a dark band on the film. Only plasmid-encoded proteins will be detected on the autoradiogram.

In this experiment, we will perform a maxi-cell analysis on the plasmid carrying the *purA* gene both before and after mutagenesis. The protein products will be separated by SDS-PAGE, and the radioactive proteins will be viewed by autoradiography. In this way we will confirm that pMB1 encodes the PurA protein, and we will determine if the Kan cartridge mutagenesis of the gene was successful.

Materials

Media

K broth; Hershey broth; Hershey salts

Reagents

³⁵S-methionine (*Caution:* radioactive! See manufacturer's instructions.); 100 mM NaCl; 15 mg/ml cycloserine; commercial molecular weight standard proteins (Bio-Rad); 30% acrylamide monomer stock solution; resolving gel buffer (1.5M Tris-Cl, pH 8.8); stacking gel buffer (0.5M Tris-Cl, pH 6.8); sterile distilled water; 10% SDS; TEMED; 10% ammonium persulfate, freshly made; SDS-PAGE running buffer (pH 8.3); 1X Sample Buffer; Coomassie blue stain (0.1% Coomassie blue R250 in 40% methanol, 10% acetic acid); glacial acetic acid; methanol.

Equipment and Supplies

SDS-PAGE electrophoresis apparatus; power supply; spectrophotometer (such as a Bausch and Lomb Spectronic 20); 37° C incubator; 37° C shaking water bath; GE 15-watt germicidal bulbs (stock number G15T8); sterile culture tubes; sterile petri dishes or multiple-well tissue culture dishes; microcentrifuge tubes; microcentrifuge; gel-drying equipment; X-ray film (Kodak X-Omat AR Film, 8 × 10 in); Kodak X-ray exposure holder, 8 × 10 in; film-developing equipment and chemicals; disposable gloves; disposable serological pipettes

Cultures

Transformation plates stored at 4° C (HA3/pMB2, HA3/pMB3, and HA3/pBR322); TY (adenine) plate culture of *E. coli* HA3 grown overnight at 37° C.

Procedure

The cultures used in this experiment must be grown and irradiated in advance of the laboratory period. This can be performed by the students or by the instructor, depending on individual preference.

Two days before the lab period:

1. Subculture one colony from each of the transformation plates (HA3/pMB2, HA3/pMB3, HA3/pBR322) to three tubes containing 1 ml of K broth. Also subculture *E. coli* HA3 (no plasmid) to a fourth tube of K broth.
2. Incubate at 37° C overnight.

One day before the lab period:

1. Subculture 0.1 ml from each culture to four sterile 13- \times -100-mm glass test tubes containing 2.9 ml of K broth. Incubate in a shaking 37° C waterbath until the cell concentration increases to approximately 2×10^8 cells/ml. This can be estimated by determining the cell density spectrophotometrically. Periodically remove the tubes from the waterbath and measure the absorbence of the culture at 600 nm. The concentration of the culture is approximately 2×10^8 cells/ml when the A_{600} equals 0.5.
2. When the cells reach the appropriate density, transfer 2.5 ml from each culture to separate sterile petri dishes (or separate wells in a 6-well tissue culture plate). Remove the covers and irradiate the cells for 25 seconds under two GE 15-watt germicidal bulbs at a distance of 50 cm above the petri dishes. This represents a UV dose of 50 J/m².
3. Add 15 μ l of cycloserine (15 mg/ml) to four sterile test tubes. Transfer 2 ml from each irradiated culture to the tubes containing the cycloserine and mix.
4. Incubate the cultures in a shaking 37° C waterbath overnight.

Day of the lab:

1. Add 1.5 ml from each of the irradiated cultures to separate microfuge tubes. Spin on high speed for two minutes at room temperature. Pour off and discard the supernatant.
2. Wash the pellets by adding 1 ml of Hershey salts to each pellet. Completely resuspend the cells, then spin for two minutes. Pour off and discard the supernatant.
3. Repeat the wash with 1 ml of Hershey salts. Resuspend the pellet in 0.8 ml of Hershey broth, and incubate in a 37° C waterbath for one hour. (During this incubation period, prepare the acrylamide gel.)
4. Add 0.2 ml of Hershey broth containing 5 μ Ci of ³⁵S-methionine to each tube. (Follow manufacturer's instructions to determine the correct concentration.) Incubate for two hours in a 37° C waterbath.
****Note:** ³⁵S is a radioactive isotope. Users should wear gloves and a lab coat at all times and exercise extreme caution when working with it. All waste generated following this step is radioactive and must be disposed of properly.
5. Centrifuge the tubes for two minutes to pellet the cells. Discard the supernatant in a vessel that can accommodate radioactive waste, such as a properly labeled beaker or jar.
6. Resuspend the pellets in 1 ml of 100 mM NaCl. Spin to pellet cells, then remove and discard the supernatant in the radioactive waste.
7. Resuspend the pellets in 50 μ l of 1X Sample Buffer in preparation for SDS-PAGE. Boil the samples for five minutes to lyse the cells and denature the proteins. Once the samples have been boiled, hold the samples on ice until they can be loaded onto the gel. Also prepare the molecular weight standards according to manufacturer's instructions.

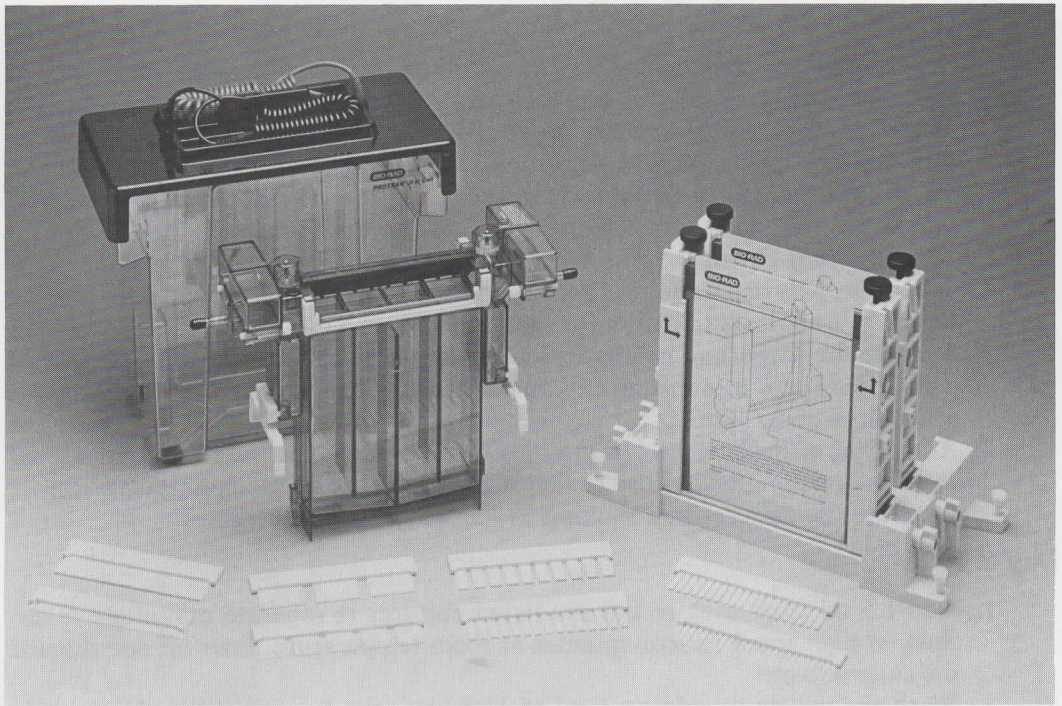


Figure 9.4. Bio-Rad's Protean II electrophoresis apparatus. Courtesy of Bio-Rad Laboratories.

A. SDS-PAGE

We will be performing SDS-PAGE using the Laemmli discontinuous buffer system. In this system, two gels that differ in pH are employed. Chloride ions and glycine ions in the running buffer migrate through the gel when an electric field is applied. As these ions migrate through the stacking gel, the protein samples become sandwiched between the two ion fronts. When the protein samples reach the resolving gel at a higher pH, the mobility of the ions changes and the sandwich dissipates, allowing the proteins to separate according to their size. Use of a stacking gel greatly enhances the resolution of proteins in the resolving gel.

****Note:** Acrylamide in solution is toxic. Therefore, use gloves when working with it.

1. Set up the electrophoresis apparatus according to manufacturer's instructions.
2. Prepare a 12% acrylamide resolving gel. To a sidearm Erlenmeyer flask, add:

10 ml distilled water
7.5 ml resolving gel buffer
12 ml 30% acrylamide stock

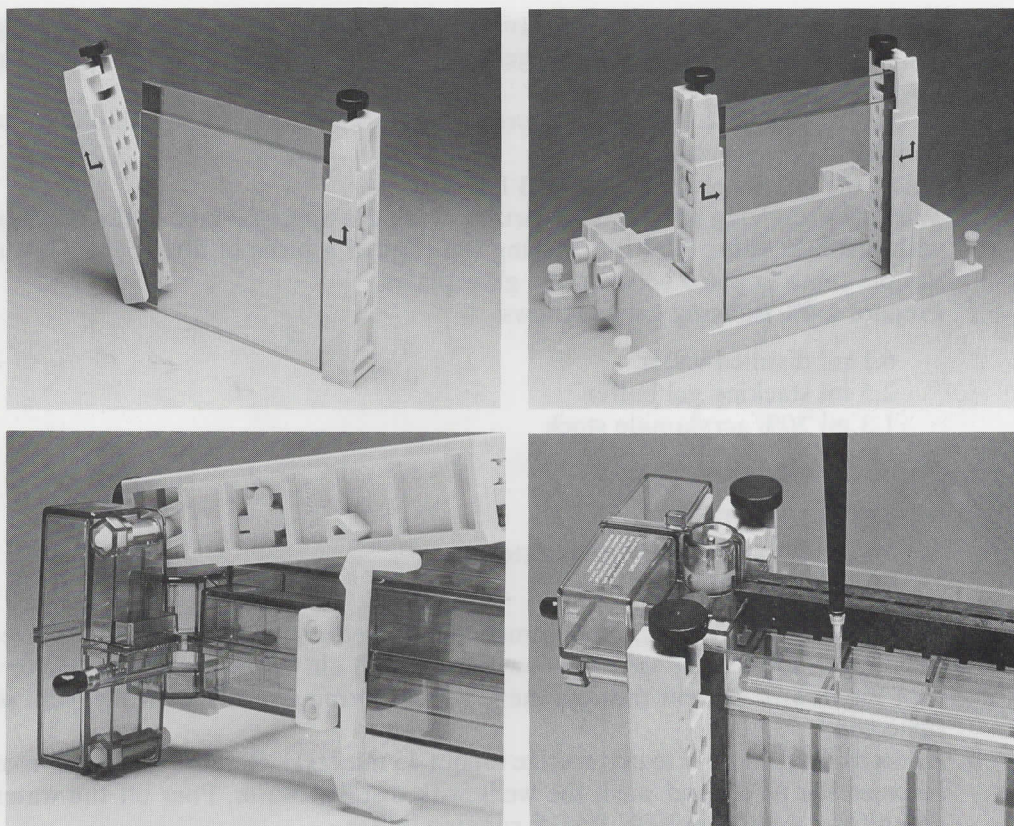


Figure 9.5. Assembly of an SDS-PAGE apparatus (the Bio-Rad Protean II system)

(1) The glass plates are assembled to form the gel sandwich. (2) The gel sandwich is placed into the casting stand, and acrylamide solution is pipetted in between the plates. (3) The gel sandwiches are assembled and placed into the apparatus to form the buffer chambers. (4) Samples are loaded into the preformed sample wells. Courtesy of Bio-Rad Laboratories.

Place a rubber stopper on the flask and degas the solution by attaching it to a vacuum pump or aspirator for fifteen minutes. Detach the flask from the vacuum and add:

300 μ l 10% SDS

150 μ l 10% ammonium persulfate (made fresh)

15 μ l TEMED

Swirl the solution gently. This recipe makes 30 ml of solution, which is adequate for use with many types of electrophoresis apparatuses.

3. Immediately pipette the solution between the glass plate of the apparatus, leaving sufficient room to pour a stacking gel on top (two to three inches, depending on the apparatus).
4. Overlay the gel with resolving gel overlay to prevent oxygen from inhibiting the polymerization reaction.
5. Allow the gel to polymerize (2 to 3 hours).
6. When the resolving gel has polymerized, wash the upper surface of the gel once with distilled water. Blot it dry using the edge of a piece of filter paper. Put a sample comb in place between the glass plates.
7. Prepare a 4% stacking gel as follows:

- 6.1 ml distilled water
- 2.5 ml stacking gel buffer
- 1.3 ml 30% acrylamide stock

Degas the solution, then add:

- 100 μ l 10% SDS
- 50 μ l 10% ammonium persulfate
- 10 μ l TEMED

Swirl the solution to mix, then immediately pipette onto the surface of the resolving gel. Pipette the solution slowly to prevent air bubbles, which will interrupt the flow of current through the gel, from forming underneath the teeth of the comb.

8. Allow the stacking gel to polymerize (about 45 minutes). Remove the comb from between the plates and wash the wells with distilled water. Pour off the water and fill the wells with SDS-PAGE running buffer.

B. Loading the Samples

1. Spin the boiled samples in a microfuge on high speed for one minute to pellet the cell debris.
2. Load 15 μ l from each sample into the sample wells. Load the molecular weight standards into the first lane.
3. Electrophorese the gel at 15–20 mA constant current overnight (or at 50 mA for approximately 3 hours). Note that electrophoresis time will vary, depending on the apparatus used. At this point, consult with your instructor, who may wish to complete the staining and autoradiography for you.
4. Remove the gel from between the glass plates and place it in Coomassie blue stain. Stain the gel for one to four hours with shaking.
5. Remove the gel from the stain solution and place it in Destain I (50% methanol, 10% acetic acid) for 1–2 hours. Transfer the gel to Destain II, and destain until the background of the gel is clear and the blue protein bands are visible. Change the Destain II solution frequently during this process.
6. Dry the gel down onto filter paper (requires gel drying equipment).

C. Autoradiography

1. Place a sheet of X-ray film into an X-ray exposure holder. Place the dried gel face down on top of the film, and close the pack. (This step must be done in the dark, preferably in a darkroom.)
2. Allow the exposure to continue for at least 24 hours. In the dark, open the pack and remove the X-ray film. Develop the film using standard photography techniques.

Assignment

****Note:** The dried gel still contains radioactivity and should, therefore, only be handled when wearing gloves.

1. Compare the autoradiogram to the dried gel. Determine which proteins are the result of plasmid-coded protein synthesis.
2. Prepare a standard curve from the molecular weight standards, as follows:
 - a. Measure from the top of the resolving gel to the top of each protein band (in cm) in the molecular weight standards lane. Also measure the distance from the top of the gel to the top of the Coomassie blue dye front (near the bottom of the gel).
 - b. Calculate R_f (relative mobility) for each standard protein:

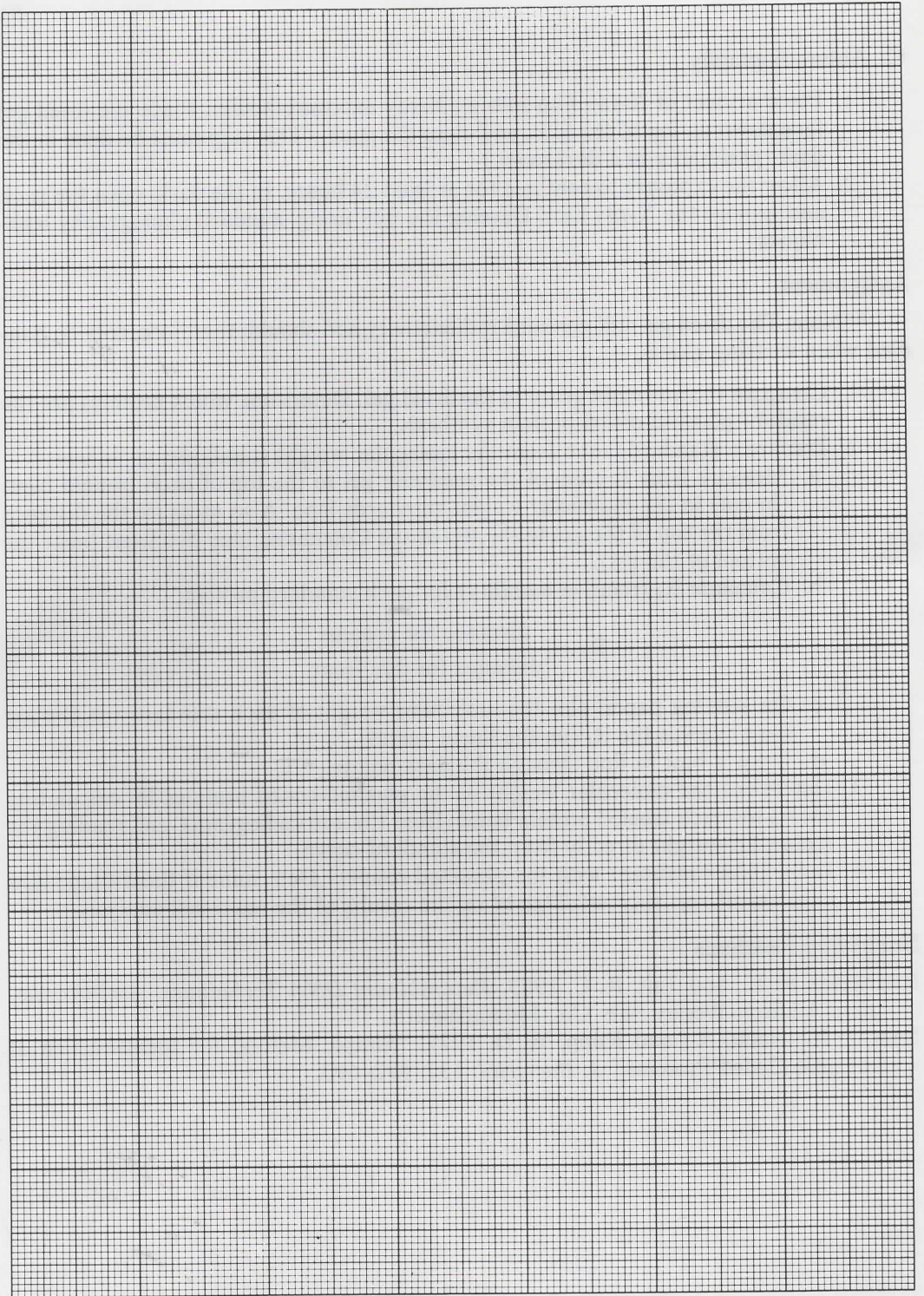
$$R_f = \frac{\text{distance for protein}}{\text{distance for dye front}}$$

- c. On semilog paper, plot the molecular weight of each standard (on the log scale) versus the R_f (on the linear scale).
 - d. Draw a best fit line through the data points.
3. Determine the molecular weight of the plasmid-coded proteins by calculating the R_f for each labeled protein band (from the autoradiogram), and reading the molecular weight directly from the standard curve.
 4. Is the data consistent with the expected results? Determine if the mutagenesis experiment was successful based on the results of the maxi-cell analysis.

Suggested Readings

Rodriguez, R. L., and Tait, R. C. 1983. *Recombinant DNA Techniques: An Introduction*. Addison-Wesley Pub. Co., Massachusetts.

Sancar, A.; Hack, A.; and Rupp, W. D. 1979. "Simple Method for Identification of Plasmid Coded Proteins." *J. Bacteriol.* 137: 692-693.



NOTES AND CALCULATIONS

Chapter 10

Isolation of High Molecular Weight (Chromosomal) DNA

Introduction

Artificial hybridization of nucleic acid probes to homologous sequences of DNA has become a powerful technique often employed in a molecular biology laboratory. Nucleic acid hybridization techniques are mostly used for the preliminary analysis of a sequence of DNA (such as genomic DNA) in order to screen for the presence of a particular gene. In this technique, chromosomal DNA is isolated from cells and is cut with restriction enzymes. This generates a large number of DNA fragments, which are then separated by agarose gel electrophoresis. The DNA is then transferred to a nitrocellulose or nylon membrane to immobilize it, and a specific, labeled nucleic acid probe is added. The probe hybridizes to homologous sequences on the chromosomal DNA. It is therefore possible to detect specific bands corresponding to the gene or DNA sequence of interest.

The first step in this process is the isolation of chromosomal DNA from organisms of interest. Cells are lysed and cellular proteins are extracted from the lysate by phenol extraction. The nucleic acids are then isolated by ethanol precipitation. RNA can be removed from the prep by treatment with *RNase*, which selectively degrades RNA and leaves DNA intact. However, high-quality chromosomal DNA is often difficult to purify, because of its bulk. Care must be taken during manipulations to prevent it from becoming sheared into many smaller pieces.

Recent advances have provided researchers with quicker and simpler means of extracting chromosomal DNA from cells. Nucleic acids carry a large negative charge, and can, therefore, be separated from other cellular components by *ion exchange chromatography*. Ion exchange chromatography is based on the principle that charged molecules in a sample will reversibly adsorb to oppositely charged molecules on a matrix. Ion exchange media consist of an insoluble matrix to which positively or negatively charged functional groups are covalently bonded. Since positively charged media bind anions, they are called *anionic exchangers*. *Cationic exchangers* are negatively charged media and bind cations. For example DEAE-cellulose, a commonly used anionic exchange medium, consists of a cellulose matrix to which the positive functional group diethyl-amino ethyl is covalently bound.

The separation of nucleic acids by ion exchange chromatography is a two-step process of adsorption and desorption, as shown in figure 10.1. Initially, counter ions from the environment bind to the positively charged matrix to maintain charge neutrality at

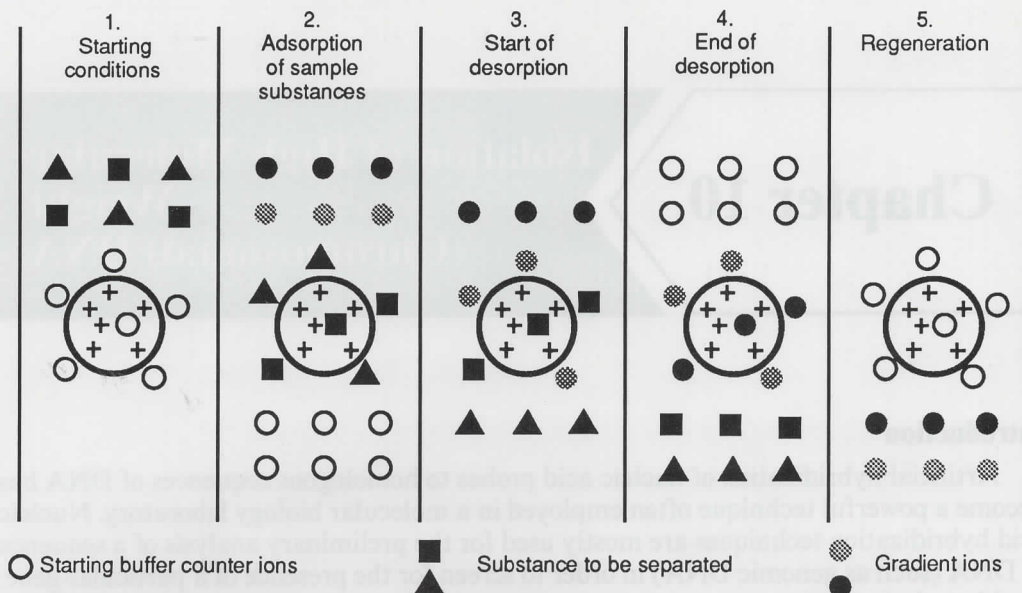


Figure 10.1. Ion exchange chromatography involves a two-step process of adsorption and desorption. From *Ion Exchange Chromatography*. Copyright © Pharmacia Fine Chemicals, a division of Pharmacia Inc., Piscataway, NJ.

the surface of the medium. When lysed cells are added, the counter ions are exchanged for the negatively charged nucleic acid molecules, which because of their greater affinity, will adsorb selectively to the matrix. Other cellular components do not bind to the column and are washed out. The nucleic acid molecules remain bound to the column.

The removal of the bound molecules is completed in the second step, desorption. A buffer of high ionic strength (a pH gradient can also be used) is applied to the column. The change in ionic strength alters the strength of the electrostatic interactions between the nucleic acid molecules and the exchange media. Desorption occurs when the DNA/RNA is exchanged for negatively charged counter ions in the eluting buffer. After the nucleic acids have desorbed, they are eluted from the column and collected. The RNA can be removed from the eluate by RNase treatment, and the DNA can be concentrated by ethanol precipitation.

In this experiment, nucleic acids from the prokaryotes *E. coli*, *Salmonella typhimurium*, *Bacillus subtilis*, and *Micrococcus luteus* will be isolated by ion exchange chromatography, utilizing the Extractor System from Molecular Biosystems. The chromosomal DNA will be concentrated by ethanol precipitation and will be stored at 4° C, to be screened for DNA sequences homologous to the *purA* gene of *E. coli*.

Materials

Media

Brain Heart Infusion (BHI) broth

Reagents

The Extractor (kit from Molecular Biosystems, Inc.) includes anion exchange columns, specimen dilution reagent, lysing reagent, wash reagents, high-salt elution reagent; proteinase K 20 mg/ml; 95% ethanol; 70% ethanol; 5M NaCl; TE pH 8.0; 50X TAE; agarose; ethidium bromide 10 mg/ml; purified λ DNA, 6X Sample Buffer

Supplies and Equipment

Rack for holding column; sterile 5-ml serological pipettes; 10-cc plastic, disposable syringe; micropipettors and tips; microfuge; microfuge tubes; ice; 60° C waterbath; mini-gel apparatus; power supply

Cultures

BHI broth cultures of *E. coli* K-12 (wild type), *Salmonella typhimurium* LT2, *Micrococcus luteus*, and *Bacillus subtilis*, grown overnight at 37° C

Procedure

In this experiment, isolation of genomic DNA using the Extractor System (Molecular Biosystems) is described. The conventional method for genomic DNA preparation is described in appendix V. It is recommended that students break into groups, with each group preparing DNA from a different cell type.

A. Preparation of the Cells

1. Pellet the cells in the overnight cultures by centrifugation.
2. Remove the supernatant by aspiration, leaving the cell pellet as dry as possible. Resuspend the cells in 1 ml of Specimen Dilution reagent.
3. Add 100 μ l of proteinase K (20 mg/ml) to the resuspended cells and mix thoroughly.
4. Add 1 ml of Lysing reagent to the solution and mix thoroughly.
5. Incubate the mixture for thirty minutes at 60° C.

B. Preparation of the Column

1. Remove the top cap from the column. (The top cap must be removed first.)
2. Remove the cap from the bottom of the column. Place the column in a rack and allow it to drain completely. If a white disk is visible above the top of the column bed, gently push it back down with the wide end of a Pasteur pipette until it just touches the top of the bed, but do not compress the bed. The flow will automatically stop when the liquid level reaches the disk.

C. Extraction of Nucleic Acids

1. Remove the lysed cells from the 60° C water bath and apply the entire sample to the top of the column with a Pasteur pipette.
2. Place a 10-cc syringe into the top of the column to create a tight fit. Using the plunger, slowly push the sample onto the column bed. (Do not exceed a rate of 20 drops per minute, or the nucleic acids will not bind efficiently.) At no time should the column bed be allowed to run dry.
3. After the sample has run completely onto the column bed, add 3 ml of Wash Reagent 1 to the top of the column. Push it through slowly (20 drops/minute, do not allow the bed to run dry). Collect the eluate in a waste beaker.
4. Add 3 ml of Wash Reagent 2 to the column and slowly push it through. (Do not allow the bed to run dry.) Collect the eluate in a waste beaker.
5. Add 2 ml of Elution Reagent to the column and slowly push it through. The eluate contains the genomic DNA. Collect 0.5 ml fractions in four microfuge tubes.

D. Ethanol Precipitation

1. To each tube, add 1/10 volume of 5 M NaCl and 2 volumes of cold 95% ethanol. Incubate the tubes on ice for at least one hour, or 15 minutes at -70° C. (If lab time is running short, the tubes can be kept in a freezer for up to one week.)
2. Centrifuge the tubes at 4° C for twenty minutes. Decant the supernatant and gently tap the inverted tubes over a paper towel to drain off the liquid.
3. Add 1 ml of 70% ethanol to the pellets and flick the tubes with your fingers once or twice. Centrifuge for fifteen minutes at 4° C. Decant the supernatant and completely drain the tubes.
4. Dry the nucleic acid pellets completely.
5. Add 25 μ l of TE pH 8.0 to each tube and allow them to stand at room temperature for ten to fifteen minutes.
6. Using a micropipettor, gently resuspend the DNA/RNA in each tube. When the pellet is completely resuspended, combine the contents of the four tubes into one and label the tube according to the organism from which it came.

E. Estimation of DNA Concentration

1. While the DNAs are precipitating, prepare a 0.7% agarose mini-gel and 250 ml of running buffer (1X TAE).
2. Prepare concentration standards by diluting stock λ DNA to 5 μ g, 1 μ g, and 0.1 μ g in 10 μ l of distilled water.
3. Remove a 5- μ l aliquot from each genomic DNA preparation. Add 5 μ l of distilled water to each tube and mix.
4. Add 2 μ l of 6X Sample Buffer to each tube. Spin the tubes for five seconds in a microfuge.

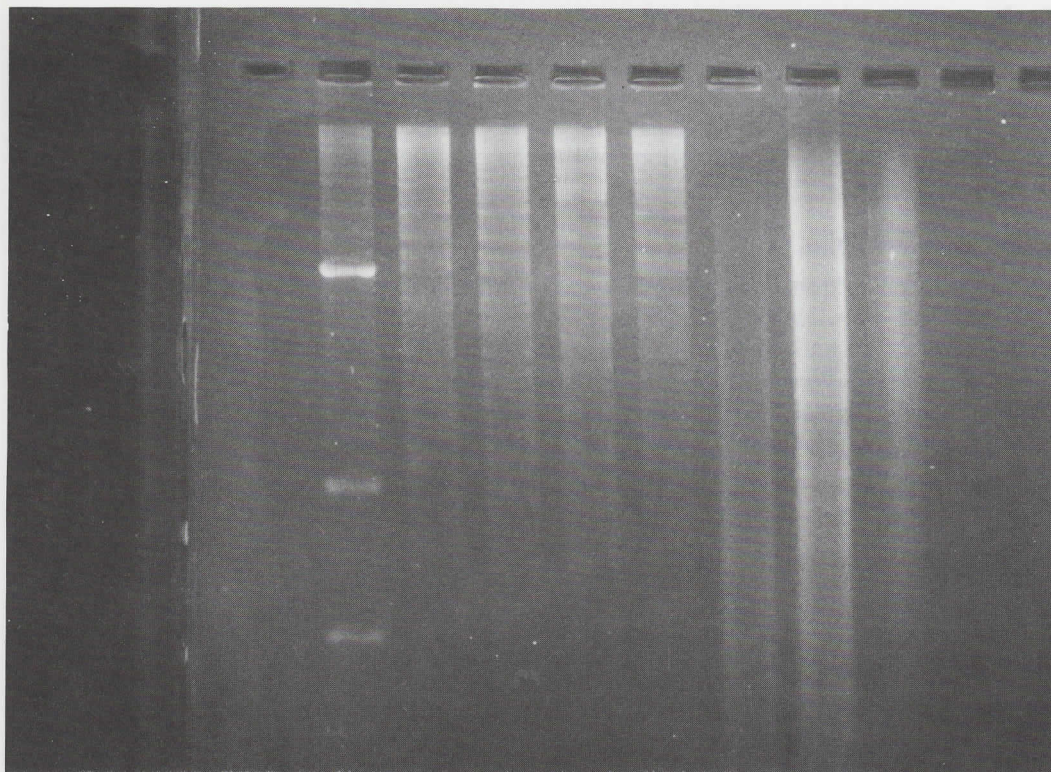


Figure 10.2. Lane 1 shows plasmid DNA digested with EcoR I. Lanes 2–5 contain genomic DNA digested to completion with EcoR I. Lanes 6–8 contain genomic DNA that was “sheared” during the preparation process.

5. Load the standards and samples onto the agarose gel. Electrophorese at 100 volts until appropriate migration has been achieved.
6. Place the gel on a UV light box. A photograph should be taken to provide a permanent record. An estimation of the chromosomal DNA concentration can be made directly from the gel or from the photograph.
7. Carefully examine the chromosomal DNAs. The DNA should appear as a discrete band on the gel. If the chromosomal DNA is sheared into small pieces, it will appear as a bright “smear” on the gel. Sheared DNA (figure 10.2) can not be used in the following Southern blot procedure, and the process should be repeated for any sheared samples.
8. Label the tubes with the type of DNA and the estimated concentration. Store all of the samples at 4° C.

Assignment

1. Discuss the principles of ion exchange chromatography as they apply to the isolation of high molecular weight DNA from biological specimens. Compare the method used in this experiment to the traditional methods.
2. Estimate the concentrations of the the chromosomal DNA obtained from each preparation.

Suggested Readings

The Extractor System, product information and instructions from Molecular Biosystems, Inc. San Diego, California.

Rodriguez, R. L., and Tait, R. C. 1983. *Recombinant DNA Techniques: An Introduction*. Addison-Wesley Pub. Co., Massachusetts.

Sambrook, J.; Fritsch, E. F.; Maniatis, T. 1989. *Molecular Cloning: A Laboratory Manual, 2nd ed.* Cold Spring Harbor Laboratory Press, NY.

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Chapter 11

Southern Blotting of Genomic DNA

Introduction

In 1975, E. M. Southern developed a method that allowed him to detect specific DNA sequences in target DNA that had been digested with restriction enzymes and separated by gel electrophoresis. In his honor, this method has since been referred to as a *Southern blot*, or *Southern blot analysis*. In this technique, target DNA is first digested with restriction enzymes to generate a number of fragments. Recall from previous experiments that restriction enzymes cleave DNA every 4^n base pairs, where 4 represents the four different bases, and n is equal to the number of bases in the recognition sequence for any given restriction enzyme. For example, EcoR I has a six-base recognition sequence. Therefore, probability rules predict that EcoR I will cleave a sequence of DNA every 4^6 , or 4,096 base pairs. The frequency of cleavage for any particular molecule of DNA then is dependent on the length of the recognition sequence. The longer it is, the less likely it will occur by chance in a sequence of DNA. Small molecules of DNA, such as plasmids, are usually cut only once or a few times by a single restriction enzyme. For example, the plasmid pBR322 is 4,362 base pairs long. Since EcoR I should have a recognition sequence only once every 4,096 bases, there should be only one recognition site for EcoR I on pBR322, which there is.

More complex DNA molecules, such as genomic DNA, contain many more recognition sites and are cut into a large number of fragments of varying size. *E. coli*, for example, contains a single circular chromosome that is 4.2×10^6 base pairs in length. The enzyme EcoR I cuts once every 4,096 bases; therefore, the *E. coli* genome is cut into approximately 1,000 fragments. There are so many fragments generated from a restriction digestion of genomic DNA that the individual bands are not seen following agarose gel electrophoresis; instead, the DNA looks like a bright “smear” against the dark background of the gel. *Using a distinctly labeled nucleic acid probe, it is possible to pick out specific bands from the smear where the probe has hybridized to complementary sequences in the target DNA.*

Because DNA fragments cannot be easily handled directly in an agarose gel, the DNA must be transferred to a medium in which hybridization reactions can occur. DNA can be transferred from an agarose gel to a nitrocellulose or nylon membrane by means of a Southern blot. DNA in an agarose gel is first denatured by alkali treatment to give single-stranded DNA fragments. These fragments are then transferred to a support

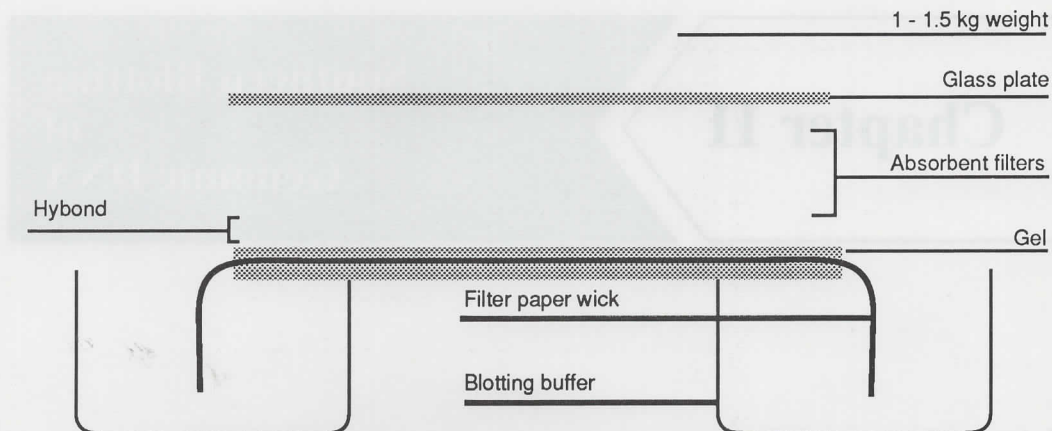


Figure 11.1. A typical capillary blotting set-up. From *Nucleic Acid Filter Hybridization*. Copyright © Amersham Corp., Arlington Heights, IL.

membrane. Because of its high binding capacity, nitrocellulose paper has been the most commonly used medium for blotting DNA, despite the fact that it was extremely fragile and difficult to handle. Nylon membranes have recently become more popular because of a greater physical strength.

Nucleic acids are transferred from a gel to a support membrane by simple capillary action. The membrane is placed on the gel, and then dry filter paper is placed on top of the membrane. A salt solution, the transfer buffer, is added to the system. The transfer buffer is attracted to the dry filter paper, passing through the agarose gel and the membrane in the process. The DNA in the gel is carried along with the salt solution, but becomes immobilized on the membrane because it is single-stranded, while the buffer passes through. A single-stranded replica of the agarose gel is thus created on the membrane.

There are three ways in which the transfer can be completed. The capillary blot was the technique first used by Southern and is still popular today because it is easy to set up and requires no specialized equipment. However, the capillary method generally takes up to 18 hours to complete, and the lengthy transfer time can result in diffusion of DNA fragments away from the membrane (see figure 11.1).

Two other methods were developed to eliminate this problem. *Electroblotting*, blotting denatured nucleic acids to support membranes electrophoretically at high current, is shown in figure 11.2. This reduces the time needed to complete the blot; however, efficient transfers require the use of high ionic strength buffers that can lead to very high electrical currents and overheating. This method also requires expensive specialized equipment that can make the process cost prohibitive for many labs.

The transfer of nucleic acids by *vacuum blotting* has become increasingly popular. The technique is similar to the capillary method, except that the transfer is vacuum driven. Buffer is drawn from an upper chamber into a lower chamber and passes through the gel and membrane, as shown in figure 11.3. The denatured DNA is transferred and

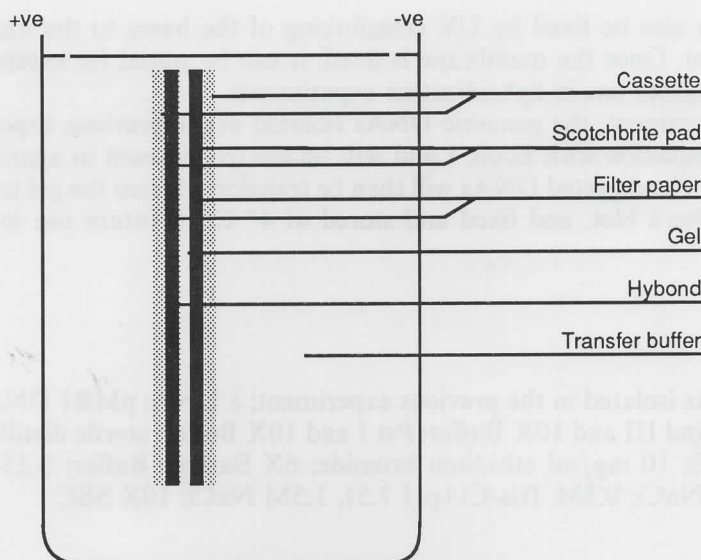


Figure 11.2. An electroblotting apparatus. From *Nucleic Acid Filter Hybridization*. Copyright © Amersham Corp., Arlington Heights, IL.

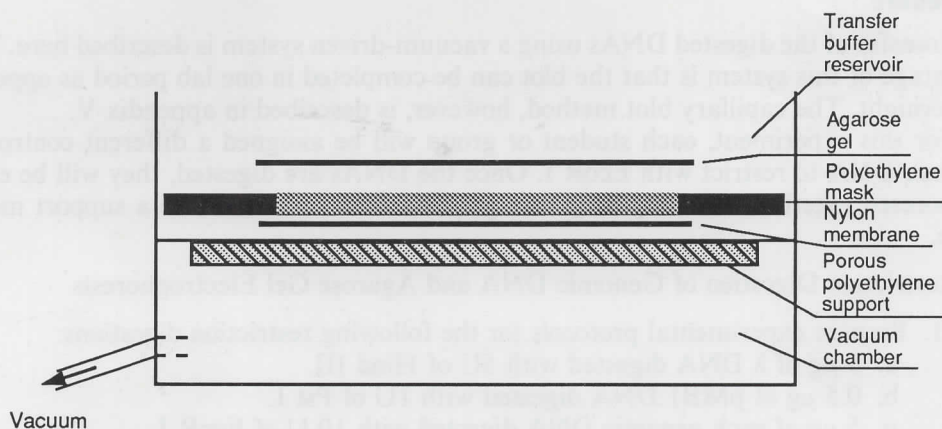


Figure 11.3. A vacuum blotting apparatus. From *Nucleic Acid Filter Hybridization*. Copyright © Amersham Corp., Arlington Heights, IL.

becomes immobilized on the membrane. The entire process can be completed in less than thirty minutes. The necessary equipment, a vacuum blotting chamber, is not expensive and the blot can be completed in a fraction of the time required for the other methods.

Once the DNA has been transferred to a suitable medium, it must be fixed to the membrane to prevent loss of the target DNA during the hybridization reaction. Usually, the membrane is baked in an oven at 80° C. If nitrocellulose is used as the support membrane, the blot must be baked under vacuum because of its combustibility. The

membrane can also be fixed by UV crosslinking of the bases to the membrane or by alkali treatment. Once the membrane is fixed, it can be stored for extended periods at 4° C for subsequent use in hybridization experiments.

In this experiment, the genomic DNAs isolated in the previous experiment will be digested to completion with EcoR I and will be electrophoresed in agarose to separate the fragments. The digested DNAs will then be transferred from the gel to a nylon membrane by Southern blot, and fixed and stored at 4° C for future use in hybridization experiments.

Materials

Reagents

Genomic DNAs isolated in the previous experiment; λ DNA; pMB1 DNA; EcoR I and 10X Buffer; Hind III and 10X Buffer; Pst I and 10X Buffer; sterile distilled water; agarose; 50X TAE; 10 mg/ml ethidium bromide; 6X Sample Buffer; 0.25M HCl; 0.5N NaOH, 1.5M NaCl; 0.5M Tris-Cl (pH 7.5), 1.5M NaCl; 10X SSC

Supplies

Nitrocellulose or nylon membrane (GeneScreen nylon membrane); VacuBlot apparatus (American Bionetics); vacuum source; 37° C water bath; mini-gel apparatus; micropipettors and tips; power supply; rubber gloves; small glass or plastic trays

Procedure

The transfer of the digested DNAs using a vacuum-driven system is described here. The advantage of this system is that the blot can be completed in one lab period as opposed to overnight. The capillary blot method, however, is described in appendix V.

For this experiment, each student or group will be assigned a different control or genomic DNA to restrict with EcoR I. Once the DNAs are digested, they will be electrophoresed together on a single agarose gel, and then transferred to a support membrane.

A. Restriction Digestion of Genomic DNA and Agarose Gel Electrophoresis

1. Prepare experimental protocols for the following restriction digestions:
 - a. 5 μ g of λ DNA digested with 5U of Hind III.
 - b. 0.5 μ g of pMB1 DNA digested with 1U of Pst I.
 - c. 5 μ g of each genomic DNA digested with 10 U of EcoR I.

Prepare the digestions in a total volume of 20–30 μ l. Add the reactants to labeled microfuge tubes, and incubate in a 37° C water bath for at least one hour.

2. Prepare a 0.7% agarose mini-gel and 250 ml of running buffer (1X TAE).
3. When the digestions are completed, add an appropriate amount of 6X Sample Buffer to each tube and spin briefly in a microfuge.
4. Load the digestions onto the gel and electrophorese at 80–100 volts until appropriate migration has been achieved (approximately one hour).

B. Pretreatment of the Gel

1. Following electrophoresis, carefully remove the gel from the electrophoresis apparatus and place it into a small glass or plastic tray.
2. Add enough 0.25M HCl (depurination solution) to completely cover the gel, and allow it to soak for ten minutes. Pour off and discard the solution. Add fresh depurination solution and allow the gel to soak for ten minutes more.
3. Pour off the HCl and replace it with 0.5N NaOH, 1.5M NaCl (denaturation solution). Allow the gel to soak for ten minutes, then pour it off and add fresh denaturation solution. Allow the gel to soak for ten minutes more.
4. Pour off the denaturation solution and add 0.5M Tris-Cl (pH 7.5), 1.5M NaCl (neutralization solution). Allow the gel to soak for fifteen minutes.

C. Southern Blot

1. Figure 11.4 shows the VacuBlot apparatus, a vacuum-driven blotting system. Assemble the buffer collection trough and rib assembly, then place the porous divider on top, as shown.
2. Assemble the gel stack as follows: cut a piece of filter paper and the nitrocellulose or nylon membrane to the exact dimensions of the gel. Handle the membrane only while you are wearing gloves. Soak the filter paper and membrane for five minutes in a small volume of transfer buffer (10X SSC). Center the filter paper on the porous divider, then place the membrane on top of the filter paper. Gently smooth out any bubbles with your fingers.
3. Cut a hole 1/4 inch smaller than the dimensions of the gel in the rubber gasket, and wet it with distilled water. Place the rubber gasket onto the porous divider so that it is centered over the membrane and filter paper.
4. Carefully remove the agarose gel from the neutralization buffer, and center it on top of the hole in the rubber gasket. Put the buffer reservoir lid in place and tighten the screws.
5. Fill the buffer reservoir with transfer buffer (10X SSC). Hook the port in the buffer collection trough to a vacuum source (a water aspirator is adequate) and turn on the vacuum. Allow the transfer to proceed for twenty minutes.
6. Following the transfer, carefully disassemble the apparatus. Remove the agarose gel and place it on a UV light box. If the transfer was complete, then nothing should be visible on the gel. Carefully remove the membrane and place it on a piece of dry filter paper.
7. Allow the membrane to completely air dry, then bake the blot for two hours at 80° C to fix the DNA onto the membrane. If nitrocellulose is used, the blot must be baked under a vacuum. Once the blot has been fixed, wrap it in Saran Wrap. In this manner, it can be stored at 4° C for an extended period.

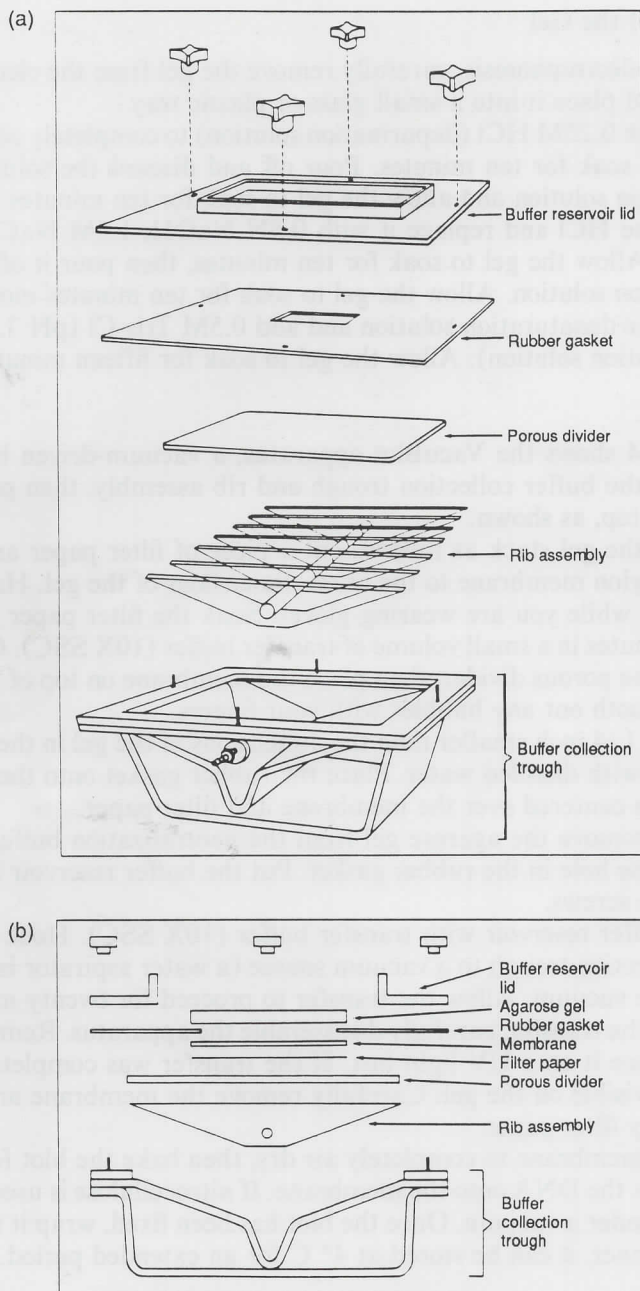


Figure 11.4. a. The VacuBlot® apparatus. b. VacuBlot buffer supply reservoir and collection trough assembly. From P. Medveczky, et al., "Rapid Vacuum Driven Transfer of DNA and RNA from Gels to Solid Supports" in *Biotechniques*, Vol. 5, No. 3:243. Copyright © 1987 Eaton Publishing Co., Natick, MA.

Assignment

1. Write an experimental protocol for the restriction digestions of the control and genomic DNAs.
2. Why must the gel be chemically pretreated before the transfer can be completed?
3. Contrast the different methods used to transfer nucleic acids to support membranes. Why is this process necessary?

Suggested Readings

- Medvecskey, P.; Chang, C. W.; Oste, C.; and Mulder, C. 1987. "Rapid Vacuum Driven Transfer of DNA and RNA from Gels to Solid Supports." *BioTechniques* 5: 242–246.
- Southern, E. 1975. "Detection of Specific Sequences among DNA Fragments Separated by Gel Electrophoresis." *J. Mol. Biol.* 98: 503–517.

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1. Write an argument for the transfer of the transfer agent to the acceptor DNA.
2. Why must the gel be thoroughly pre-washed before the transfer can be done?
3. Compare the different methods used to transfer nucleic acids to support media.
4. Name. Why is this process necessary?

Suggested Readings

- Moody, R.; Chang, C. W.; Oue, C.; and Muller, C. 1987. "Rapid Vacuum-Directed Transfer of DNA and RNA from Gels to Solid Supports." *BioTechniques* 5: 242-246.
- Southern, E. 1975. "Detection of Specific Sequences among DNA Fragments Separated by Gel Electrophoresis." *J. Mol. Biol.* 98: 503-532.

Chapter 12

Nucleic Acid Hybridization: Detection of Conserved Genes

Introduction

The DNA in a double helix can be denatured into two separate strands under appropriate conditions. This is, in fact, an intrinsic characteristic of DNA—in order for replication to occur, the strands must undergo a localized melting, or denaturation reaction, but the strands must be able to renature again quickly to prevent disruption of genetic functions. *In vitro*, the double helix can be denatured by heat or by increasing the pH of the environment. When the environmental conditions are returned to a physiological state, the single-stranded regions are able to reanneal by complementary base pairing to form duplex DNA. This property has become used extensively in nucleic acid blotting and hybridization experiments to screen genomic DNA for the presence of a specific gene, to facilitate gene isolation and mapping, and to detect genetic diseases.

Nucleic acid hybridization is often the first step toward detection of a specific gene in the genome of an organism. Genomic DNA is fractionated by restriction digestion and agarose gel electrophoresis. The DNA is then denatured and blotted to a filter. A single-stranded nucleic acid probe (either DNA or RNA) is added, which binds complementary regions of the blotted genomic DNA. Once the presence of a specific gene has been detected, the gene can be cloned from a genomic “library.”

The actual process of hybridization has two parts. Initially, the single-stranded probe encounters the single-stranded target DNA by chance. If the two strands have any complementary bases, they will form a few individual base pairs to generate a short, double-stranded region. This occurs slowly and is rate-limiting to the hybridization reaction. The remainder of the molecules then quickly anneal to one another in a zipperlike fashion to form a double-stranded hybrid molecule. Hybridization of two single-stranded nucleic acid molecules is a test for sequence homology; only closely matched molecules will be able to hybridize (see figure 12.1).

In order to fully understand the nature of hybridization reactions, it is necessary to examine the parameters affecting the denaturation and renaturation of DNA. The temperature range in which DNA denatures can be assayed spectrophotometrically in the ultraviolet range (recall that DNA absorbs strongly in this range). As DNA denatures, the optical density of the solution increases because the interactions between bases in a

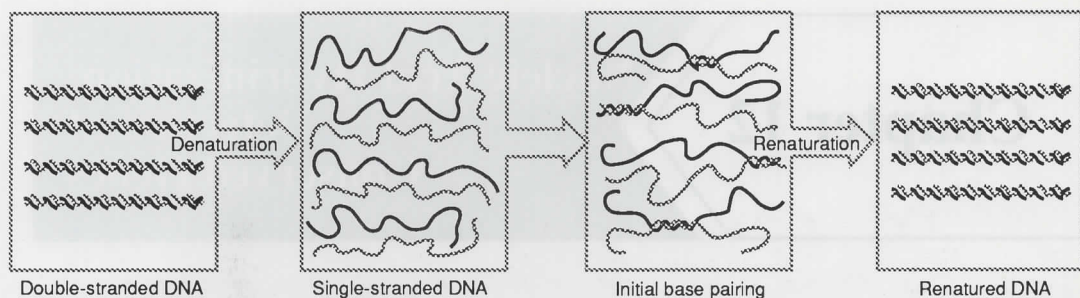


Figure 12.1. DNA denaturation and renaturation. From Benjamin Lewin, *Genes II*. Copyright © 1985 John Wiley & Sons, Inc., New York, NY.

stable double helix molecule cause a net decrease in the amount of light that is characteristically absorbed by each individual base. This is referred to as the *hyperchromic effect*; denatured DNA absorbs more strongly than double-stranded DNA. A typical “melting,” or denaturation, curve for DNA is shown in figure 12.2. The temperature at which two strands of duplex DNA are 50% dissociated is termed the T_m (*melting temperature*). The T_m is affected by environmental conditions (temperature and ionic strength), the length of the strands, and the base composition of the DNA. G-C base pairs, with three hydrogen bonds, are more stable than A-T pairs, which have two. Therefore, strands of DNA that contain a large number of G-C base pairs will melt at a higher T_m than those with a lower G-C number because more energy must be added in order to melt G-C-rich DNA. Artificial hybridization reactions are carried out at temperatures below T_m and at high ionic strength, which favors the formation and maintenance of a hybrid duplex between probe and target DNA.

The parameters affecting artificial hybridization have been researched in order to optimize the practical use of the technique. Several factors have been found to be important. The nature of the probe, in terms of its length, base composition, and the degree of homology shared with the target DNA, is very important. The concentration of probe and target DNA relative to one another is also important. In most genomic DNA blotting experiments, the nucleic acid probe concentration is in excess of target DNA to maximize the hybridization reaction rate. The length of the probe generally has very little effect on the hybridization rate when probe DNA is in excess of target DNA. However, longer probes may have sequence homology with nontarget sequences and may increase the nonspecific background of the blot.

The degree of homology between probe and target can be estimated by performing the hybridization reactions at a particular “stringency.” The term *stringency* simply refers to the specificity of the hybridization reaction between target and probe. At high stringency, only completely homologous sequences will hybridize, whereas at low stringency, sequences with less than complete complementarity will hybridize. Stringency is determined by the conditions under which the reaction is carried out. The hybridization reaction is performed under conditions that favor the formation of hybrids, usually at

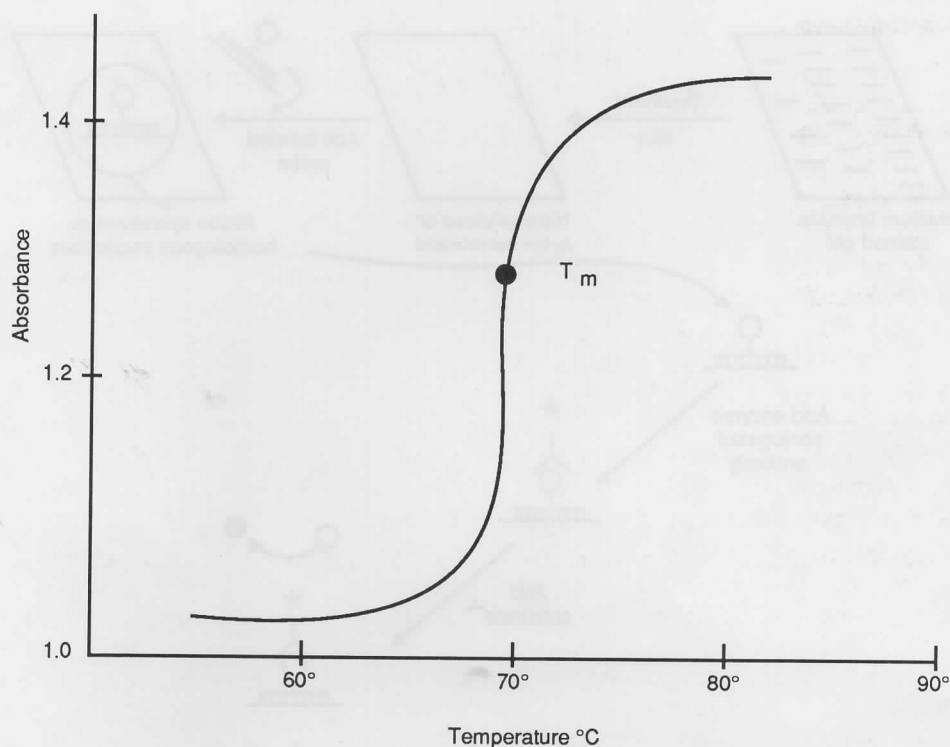


Figure 12.2. A typical DNA melting curve.

temperatures well below T_m and at high-salt concentrations. The posthybridization washes determine the stringency of the experiment. Washing the blots with a low ionic strength buffer at high temperatures will favor the denaturation of all but the most closely matched, stable hybrids. When hybridization is detected at high stringency, the probe and target DNA must be nearly completely complementary. If hybridization is not detected at high stringency but is detected at lower stringency (higher salt, lower temperature), the two DNAs are not completely homologous but contain mismatched base pairs.

In order to insure the success of a nucleic acid hybridization experiment, the probe must be properly labeled. Probes can be labeled with a radioactive isotope or with a number of recently developed nonradioactive systems. The isotope phosphorus-32 (^{32}P) is used most frequently for radioactive labeling. Hybridization with a radio-labeled probe is detected following autoradiography. ^{32}P emits β -particles, and, therefore, extreme caution must be used when working with it. Working with any radioisotope requires the use of specialized equipment and skills. To circumvent some of the dangers associated with radioactive probes, nonradioactive labeling methods were developed. Nucleic acid probes can be labeled with biotin, a vitamin. Streptavidin binds to biotin very strongly, thus

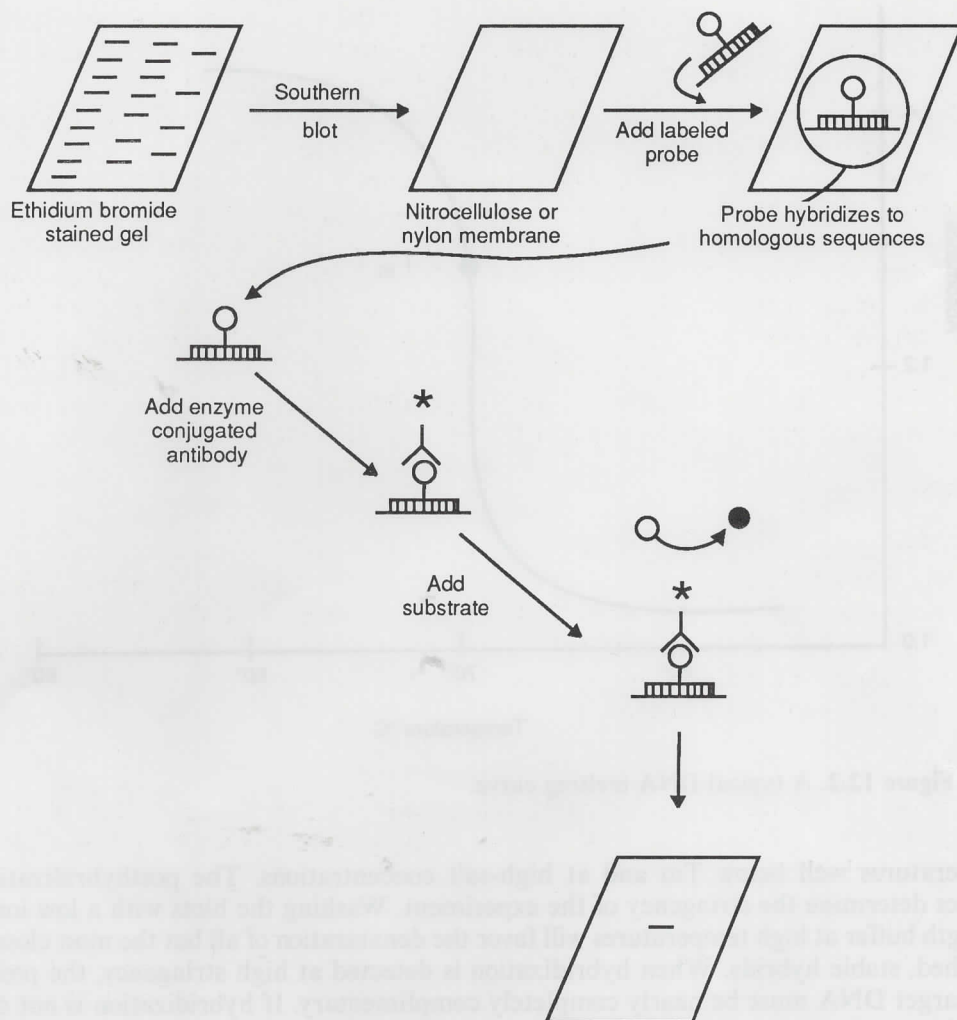


Figure 12.3. Southern blot using nonradioactive detection of hybridization.

hybridization can be detected utilizing a streptavidin-biotinylated enzyme complex. Following addition of a chromogenic substrate for the enzyme, sites of hybridization can be detected by the conversion of the colorless substrate to a colored end product. Other nonradioactive labels include sulfonation of DNA and labeling with digoxigenin. Sulfonated or digoxigenin labeled probes are detected following hybridization using enzyme-labeled antibodies (see figure 12.3).

How is the label incorporated into the probe? Two general labeling methods have been shown to work well with both radioactive and nonradioactive labels. *Nick translation* involves the use of two enzymes, DNase I and DNA polymerase. DNase I nicks the DNA, leaving nicks at random sites along the probe. DNA polymerase then repairs

the nicks by chewing away at the free ends and, at the same time, filling in with new nucleotides, using the complimentary strand as a template. The incorporated nucleotides carry a label, thus as the nick is "translated," the probe becomes labeled.

The second method is *random primer labeling*. The probe is denatured by boiling, and then a randomly generated mixture of hexanucleotides is added, along with DNA polymerase I (Klenow fragment). The hexanucleotides will pair with complimentary sequences on the probe. Klenow enzyme of DNA polymerase then uses the free 3'-OH ends on the primers to synthesize new regions to fill in the gaps between hexanucleotides using labeled nucleotide triphosphates, resulting in a labeled probe (see figure 12.4).

In this experiment, a fragment containing the cloned *E. coli purA* gene will be labeled with digoxigenin using the random primer method, and will be used as a probe to screen for homologous sequences within the genomic DNAs on the Southern blot prepared in the previous experiment. Following hybridization, the blot will be examined to determine the degree of conservation of the *purA* gene in nature.

Materials

Reagents

20X SSC; 10% N-Lauroylsarcosine (w/v); 0.2M EDTA pH 8.0; 4M LiCl; 95% ethanol; 70% ethanol TE pH 8.0; 10% SDS; 100mM Tris-Cl, 150mM NaCl, pH 7.5; 100mM Tris-Cl, 100mM NaCl, 50mM MgCl₂, pH 9.5; pMB1 DNA; Pst I and 10X reaction buffer; sterile distilled water; Genius Non-radioactive DNA Labeling and Detection Kit (Boehringer Mannheim Biochemicals)

Supplies

Microfuge; microfuge tubes; micropipettors; sterile tips; Southern blot from previous week; small, sealable plastic boxes; ice; 37° C waterbath; 65° C shaking waterbath; boiling waterbath

Procedure

To obtain the best results, the blot should be allowed to hybridize overnight. However, if the probe is used in excess, hybridizations of one hour may give acceptable results. It is also possible to allow the hybridization to react for up to one week, and the stringency washes and detection process completed at that time. It is recommended that the optimal hybridization conditions be worked out for each laboratory prior to beginning this experiment.

The labeling process used in this experiment is the Genius Labeling and Detection Kit from Boehringer Mannheim Biochemicals. The method is random primer labeling of linearized pMB1 with digoxigenin. Following the hybridization, digoxigenin-labeled sequences are detected using an anti-digoxigenin antibody coupled to an enzyme. A chromogenic substrate for the enzyme is added, and DNA fragments on the Southern blot that have sequence homology to the *purA* gene appear as dark blue bands on the membrane.

Other random primer labeling kits and nonradioactive labels work equally well and can be substituted for the method described here.

Linear denatured DNA

+ random
hexanucleotides

+ dATP, dCTP, dGTP, dTTP + Dig-dUTP
+ Klenow enzyme →

Synthesis of labeled DNA

Filter-bound
homologous DNA
+ labeled DNA

Hybridization

+ antibody conjugate
Dig AP
Antibody hapten
complex

+ X-phosphate + NBT

Color reaction

purple/blue color

Figure 12.4. The Genius Non-radioactive DNA Labeling and Detection Kit. From *Genius Product Information*. Copyright © Boehringer Mannheim Biochemicals, Indianapolis, IN.

A. Prehybridization

The baked membrane must be prehybridized prior to the hybridization reaction to block nonspecific binding of the probe. Before beginning the prehybridization, check with your instructor who may wish to complete the process for you.

Twenty-four hours before the scheduled lab period, prepare 100 ml of hybridization buffer (5X SSC, 0.1% N-Lauroylsarcosine, 0.02% SDS; 0.5% Blocking Reagent). To prehybridize the membrane, add 50 ml of the buffer to a small, sealable plastic box. Store the remainder of the solution at 4° C for use in the hybridization reaction. Slowly lower the membrane into the solution at a 45° angle so that it becomes uniformly wetted. Cover the box, and then place it in a 65° C waterbath with a weight on the cover. Allow the filter to prehybridize overnight with agitation.

Hybridization Solution:

- 25 ml of 20X SSC
- 1 ml of 10% N-Lauroylsarcosine
- 0.2 ml of 10% SDS
- 0.5 g of Blocking Reagent
- 73.3 ml of distilled water

Prepare this solution at least one hour before use. Heat to 50°–70° C to dissolve the blocking solution.

B. Preparation of the Probe

1. Write an experimental protocol for the digestion of 2 μ g of pMB1 DNA with 4 U of Pst I in a total reaction volume of 10 μ l. Add the reactants to a microfuge tube, and incubate the digestion for at least one hour at 37° C.
2. Denature the DNA and inactivate the restriction enzyme by boiling the DNA for ten minutes. Remove the tube from the boiling water bath and immediately place it on ice.
3. Add the following reagents to the DNA solution:

- 2 μ l hexanucleotide mixture
- 2 μ l dNTP labeling mixture
- 5 μ l sterile distilled water
- 1 μ l Klenow fragment

Incubate the tube at 37° C for at least one hour. The reaction can be incubated overnight if desired, as longer incubation times increase the amount of labeled DNA.

4. To stop the labeling reaction, add 2 μ l of 0.2M EDTA to the tube and mix well.
5. Add 3 μ l of 4M LiCl and 10 μ l of glycogen solution (2 mg/ml) to the probe solution and mix completely. Add 100 μ l of cold 95% ethanol to precipitate the labeled probe DNA. Incubate the tube for two hours at –20° C (or thirty minutes at –70° C).
6. Centrifuge for twenty minutes at 4° C to pellet the DNA. Wash the pellet once with cold 70% ethanol, then recentrifuge to pellet the DNA.

7. Remove the ethanol and tap the tube over a paper towel to drain off all of the liquid. Dry the pellet completely.
8. Dissolve the pellet in 50 μ l of TE pH 8.0 containing 0.1% SDS. Incubate the tube at 37° C for ten minutes with frequent mixing to completely resuspend the DNA.

C. Hybridization

1. If the hybridization buffer appears cloudy, heat it for a short time at 50° C to redissolve any precipitated material.
2. Add the labeled probe solution to 20 ml of hybridization buffer. (The approximate final concentration of the probe is 25 ng per ml of hybridization buffer.)
3. Pour off and discard the prehybridization buffer from the blot, and replace it with hybridization buffer containing the labeled probe. Incubate the blot in a 65° C waterbath overnight (or up to one week, depending on the timing).

D. Stringency Washes

1. Following hybridization, carefully remove the blot from the hybridization buffer. Add 50 ml of 2X SSC, 0.1% SDS to a second small plastic box, and slowly lower the membrane into the wash solution. Incubate the blot with agitation at room temperature for five minutes. Pour off the wash and replace it with 50 ml more of the wash solution. Agitate for five minutes.
2. Pour off the first wash solution and replace it with 50 ml of 0.5X SSC, 0.1% SDS. Incubate the blot with agitation at 65° C for 15 minutes, then pour off the wash and repeat.

E. Immunological Detection of Hybridization

1. Briefly rinse the blot in 50 ml of Buffer 1 (100mM Tris-Cl, 150mM NaCl, pH 7.5).
2. Add 100 ml of Buffer 2 (0.5% Blocking Reagent in Buffer 1) to the blot, and incubate at room temperature for thirty minutes.
3. Pour off Buffer 2, and again briefly rinse the blot in Buffer 1.
4. Dilute the antibody-conjugate 1:5,000 in 25 ml of Buffer 1 (5 μ l of conjugate in 25 ml of buffer).
5. Add the diluted antibody-conjugate solution to the blot, and incubate with gentle agitation for thirty minutes.
6. Wash the blot in 100 ml of Buffer 1 for 15 minutes. Repeat this wash once.
7. Place the blot into 25 ml of Buffer 3 (100mM Tris-Cl, 100mM NaCl, 50mM $MgCl_2$, pH 9.5) for two minutes to equilibrate the membrane, then pour off and discard Buffer 3.
8. Prepare 20 ml of color solution by adding 90 μ l of NBT solution and 70 μ l of X-phosphate solution to 20 ml of Buffer 3. Add the color solution to the blot and seal the plastic box. Incubate the blot for at least one hour. If the bands are still faint, allow the color reaction to continue overnight.

9. When the intensity of the bands is acceptable, stop the reaction by washing the membrane for five minutes in 50 ml of TE pH 8.0.
10. Examine the blot for bands that indicate sequence homology to the *purA* gene of *E. coli*. The blot can be photocopied or photographed to obtain a permanent record of the experiment.

Assignment

1. Examine the blot for the appearance of bands. Do other organisms have DNA sequences homologous to the *purA* gene of *E. coli*? What does this suggest from an evolutionary point of view?
2. Examine the control DNA lane. What does this indicate about the success of the hybridization?
3. The blot was washed following hybridization under low-salt, high-temperature conditions. What does this indicate in terms of the degree of homology between the *purA* gene and any detected homologies?
4. Comment on the specificity of the hybridization reaction (remember that linearized pMB1 was used as the probe). How might the specificity of the reaction be increased?

Suggested Readings

- Boehringer Mannheim Biochemicals, product information for Genius Non-radioactive DNA Labeling and Detection Kit.
- Sambrook, J.; Fritsch, E. F.; Maniatis, T. 1989. *Molecular Cloning: A Laboratory Manual*, 2nd ed. Cold Spring Harbor Laboratory Press, NY.

NOTES AND CALCULATIONS

10. Explain the fact that the rate of polymerization is higher in the presence of a catalyst than in its absence. (10 points)

Assignment

1. Explain the fact that the rate of polymerization is higher in the presence of a catalyst than in its absence. (10 points)
2. Explain the fact that the rate of polymerization is higher in the presence of a catalyst than in its absence. (10 points)
3. Explain the fact that the rate of polymerization is higher in the presence of a catalyst than in its absence. (10 points)
4. Explain the fact that the rate of polymerization is higher in the presence of a catalyst than in its absence. (10 points)

Suggested Reading

Bookman, M. (1980). *Principles of Polymer Chemistry*. New York: McGraw-Hill.
 DNA Labeling and Detection Kit.
 Sambrook, J., Fritsch, E. F., Maniatis, T. (1989). *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.

Appendix I

Formulations for Media and Reagents

Media

TY Medium

Broth

Tryptone	10 g
Yeast extract	5 g
NaCl	10 g
1 N NaOH	2 ml
Water	to 1 liter

Autoclave at 121°C for 15 minutes. Cool and dispense.

Plate and slant media

Add 15 g of agar to the broth.

TY with 0.1% maltose

Add 5 ml of 20% maltose after media has been autoclaved and cooled.

TY with ampicillin

Add 50 mg of ampicillin after media has been autoclaved and cooled.

TY with kanamycin

Add 30 mg of kanamycin after media has been autoclaved and cooled.

TY with adenine

Add 4 ml of 5 mg/ml adenine after the media has been autoclaved and cooled

Minimal Medium (M-9 minimal medium)

10X Salts

Na_2HPO_4	70 g
KH_2PO_4	30 g
NaCl	5 g
NH_4Cl	10 g
water	to 1 liter.

Autoclave and store at room temperature.

Minimal Medium Plates

Autoclave 15 g agar in 800 ml of distilled water. After cooling, add:

10X salts	100 ml
20% glucose	20 ml
0.1 M CaCl_2	10 ml
0.1 M MgSO_4	10 ml

Adjust volume to 1 liter with sterile distilled water

Minimal Medium with Adenine Plates

Add 4 ml of 5 mg/ml adenine solution per liter before pouring plates.

Hershey Medium**Salts**

NaCl	5.4 g
KCl	3.0 g
NH_4Cl	1.1 g
$\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$	15 mg
$\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$	200 mg
$\text{FeCl}_2 \cdot 6\text{H}_2\text{O}$	0.2 mg
KH_2PO_4	87 mg
Tris base	12.1 g
water (double distilled)	to 1 liter.
Autoclave and cool. Adjust pH to 7.4 with HCl.	

Broth

Hershey salts	100 ml
20% glucose	2 ml

K-Broth

10X M-9 salts	100 ml
20% glucose	20 ml
0.1 M CaCl_2	10 ml
0.1 M MgSO_4	10 ml
20% casamino acids	50 ml
0.1% thiamine HCl	0.1 ml
water	to 1 liter.

TB Top Agar

Tryptone	10 g
NaCl	5 g
agar	8 g
water	800 ml

Autoclave and cool. Add:

1 M MgSO_4	10 ml
---------------------	-------

Dispense into sterile tubes and store at 4° C. To use, melt agar in microwave or boiling water bath.

BHI Medium (Brain-Heart Infusion)

Brain-Heart Infusion Broth (BBL)

	37 g
water	to 1 liter

Autoclave and store at 4° C.

Media Supplements (Filter sterilize)

20% maltose in distilled water

40% glucose in distilled water

0.1% thiamine (HCl) in distilled water

Adenine, 5 mg/ml

adenine	50 mg
1 N HCl	0.5 ml
water	9.5 ml

20% casamino acids in distilled water

Reagents

1 M MgSO_4

MgSO_4	12.04 g
water	to 100 ml

Autoclave. To prepare 0.01 M MgSO_4 , dilute 1 M 1:100.

5 M NaCl

NaCl	292.2 g
water	to 1 liter

Stir to dissolve salt, then dispense into bottles. Autoclave.

100 mM CaCl_2

CaCl_2	14.72 g
water	to 1 liter

Stir to dissolve, then dispense into bottles. Autoclave.

500 mM EDTA

disodium EDTA-2H₂O 186.1 g

water 800 ml

Stir using a magnetic stirrer. Adjust pH to 8.0 with NaOH (approximately 20 g). Bring volume to 1 liter with distilled water.

Dispense into bottles and autoclave.

1 N NaOH

NaOH 40 g

water to 1 liter.

0.25 M HCl

Dilute concentrated HCl 1:48 in distilled water.

4 M LiCl

LiCl 16.96 g

water to 100 ml

Autoclave.

500 mM MgCl₂MgCl₂ 101.7 g

water to 1 liter

Stir to dissolve, then dispense into bottles. Autoclave.

10% N-Lauroylsarcosine in distilled water, filter sterilize.

2 mg/ml glycogen in distilled water, filter sterilize.

15 mg/ml cycloserine in distilled water, filter sterilize.

Tris buffers (using Trizma reagents from Sigma Chemical Co.)

To prepare 0.05 M solution, follow the chart below. Mix the amounts of Trizma base and Trizma HCL shown. Dissolve in water and bring to a final volume of 1 liter. To prepare 1 M solutions, multiply amounts $\times 20$.

TRIZMA[®] MIXING TABLE

1.	2.	3.	4.	5.	1.	2.	3.	4.	5.
pH at 5°C	pH at 25°C	pH at 37°C	Grams/Liter		pH at 5°C	pH at 25°C	pH at 37°C	Grams/Liter	
			Trizma [®]	Trizma [®]				Trizma [®]	Trizma [®]
			HCl	Base				HCl	Base
7.76	7.20	6.91	7.02	0.67	8.78	8.20	7.91	3.54	3.34
7.89	7.30	7.02	6.85	0.80	8.88	8.30	8.01	3.07	3.70
7.97	7.40	7.12	6.61	0.97	8.98	8.40	8.10	2.64	4.03
8.07	7.50	7.22	6.35	1.18	9.09	8.50	8.22	2.21	4.36
8.18	7.60	7.30	6.06	1.39	9.18	8.60	8.31	1.83	4.65
8.26	7.70	7.40	5.72	1.66	9.28	8.70	8.42	1.50	4.90
8.37	7.80	7.52	5.32	1.97	9.36	8.80	8.51	1.23	5.13
8.48	7.90	7.62	4.88	2.30	9.47	8.90	8.62	0.96	5.32
8.58	8.00	7.71	4.44	2.65	9.56	9.00	8.70	0.76	5.47
8.68	8.10	7.80	4.02	2.97					

From *Sigma Technical Bulletin No. 106B(11-78)*. Copyright © Sigma Chemical Co., St. Louis, MO.

20X SSC

NaCl	175.5 g
sodium citrate	88.2 g
water	to 1 liter

To prepare 10X SSC, dilute 20X 1:2 in distilled water.

TE pH 8.0 (10 mM Tris-Cl pH 8, 1 mM EDTA)

1 M Tris-Cl pH 8	1 ml
500 mM EDTA	0.2 ml
water	to 100 ml

Autoclave.

50X TAE

Tris base	242 g
glacial acetic acid	57.1 ml
500 mM EDTA	100 ml
water	to 1 liter

Do not autoclave.

10 mg/ml ethidium bromide in distilled water. Wrap the container in aluminum foil and store at 4° C. (10 mg/ml ethidium bromide can be purchased premade from Sigma).

6X Sample Buffer

0.25% bromophenol blue
0.25% xylene cyanol
30% glycerol
6XTAE

Make up in distilled water.

Phenol

Note: phenol can cause severe burns. Wear gloves when working with it. Purchase saturated phenol from Amresco, Inc. Follow manufacturer's instructions regarding use.

SEVAG

Mix 24 parts chloroform to 1 part isoamyl alcohol. Store at room temperature in a fume hood.

Mini-prep Solution I

1 M Tris Cl pH 8.0	2.5 ml
500 mM EDTA	2 ml
water	to 100 ml

Store at 4°C. Just before use, add 1.25 ml of 20% glucose and 20 mg lysozyme to 5 ml of Solution I.

Solution II

1 N NaOH	1 ml
10% SDS	0.5 ml
water	3.5 ml

Make immediately prior to use.

Solution III

5 M potassium acetate	60 ml
glacial acetic acid	11.5 ml
water	28.5 ml

Store at room temperature.

Denaturation Solution for Southern Blot (0.5 N NaOH, 1.5 M NaCl)

NaOH	20 g
NaCl	87.76 g
water	to 1 liter

Neutralization Solution for Southern Blot (0.5 M Tris-Cl pH 7.5, 1.5 M NaCl)

Tris HCl	63.52 g
Tris base	11.8 g
NaCl	87.76 g
water	to 1 liter

Reagents for SDS-PAGE

Note: Acrylamide is a neurotoxin.

1. Monomer Solution (30% acrylamide)

acrylamide	58.4 g
bis-acrylamide	1.6 g
water	to 200 ml

Filter. Store in the dark at 4°C.

2. Resolving Gel Buffer (pH 8.8)

Tris base	36.6 g
water	to 200 ml

Adjust pH to 8.8 with HCl.

3. Stacking Gel Buffer (pH 6.8)

Tris base	6 g
water	to 100 ml

Adjust pH to 6.8 with HCl.

4. 10% SDS in distilled water

5. Initiator

10% ammonium persulfate in distilled water, made just before use.

6. Resolving Gel Overlay

resolving gel buffer (Sol. 2)	25 ml
10% SDS (Sol. 4)	1 ml
water	to 100 ml

Water-saturated isobutanol may also be used as an overlay.

7. 2X Denaturation Buffer

stacking gel buffer (Sol. 3)	2.5 ml
10% SDS (Sol. 4)	4.0 ml
glycerol	2.0 ml
β -mercaptoethanol	1.0 ml
water	to 10 ml

Bromophenol blue (2 drops of 1%) may also be added for use as a tracking dye.

8. Tank Buffer

Tris base	12 g
glycine	57.6 g
10% SDS (Sol. 4)	40 ml
water	to 4 liters.

9. Coomassie Blue Stock Stain

Coomassie blue R-250	2 g
water	to 200 ml
Stir and filter. Store at room temperature.	

10. Working Stain

stock stain	62.5 ml
methanol	250 ml
glacial acetic acid	50 ml
water	to 500 ml

Stain acrylamide gels for 2–4 hours at room temperature with gentle shaking.

11. Destain I

methanol	250 ml
glacial acetic acid	50 ml
water	to 500 ml

Destain in Solution I following staining for 2–3 hours.

12. Destain II

glacial acetic acid	35 ml
methanol	25 ml
water	to 500 ml

Destain in Solution II until the background is completely clear.

To prepare a 10% acrylamide resolving gel with a 4% stacking gel:

	Resolving gel	Stacking gel
monomer stock	10 ml	1.33 ml
resolving gel buffer	7.5 ml	—
stacking gel buffer	—	2.5 ml
water	12 ml	6.1 ml
De-gas the solution under vacuum.		
10% SDS	0.3 ml	0.1 ml
10% ammonium persulfate	150 μ l	50 μ l
TEMED	10 μ l	5 μ l
	Final Volume = 30 ml	Final Volume = 10 ml

Restriction Enzyme Reaction Buffers

These buffers can be purchased with the enzyme or prepared as follows:

Low-Salt Buffer (0 mM NaCl)

10 mM Tris-Cl pH 7.5

10 mM $MgCl_2$

1 mM dithiothreitol

Medium-Salt Buffer (50 mM NaCl)

50 mM NaCl

10 mM Tris-Cl pH 7.5

10 mM $MgCl_2$

1 mM dithiothreitol

High-Salt Buffer (100 mM NaCl)

100 mM NaCl

50 mM Tris-Cl pH 7.5

10 mM $MgCl_2$

1 mM dithiothreitol

Reaction conditions for some restriction enzymes are shown on the next page.

ENZYME	0 mM NaCl	50 mM NaCl	100 mM NaCl	150 mM NaCl	ENZYME	0 mM NaCl	50 mM NaCl	100 mM NaCl	150 mM NaCl
Aat II	+	++	++	+	Hinf I	++	+++	+++	+++
Acc I	+++	+++	+	+	HinP I	+++	+++	+++	+++
Afl II	+	+++	++	++	Hpa I	+	+++	+++	+
Aha II	+	++	+++	+++	Hpa II	+++	+++	++	+
Alu I	+	+++	+++	++	Hph I	+++	+++	+++	+
Alw I	+++	+++	++	+	Kpn I	+++	+	+	+
AlwN I	++	+++	+++	+	Mbo I	++	+++	+++	+++
Apa I	+++	+++	++	+	Mbo II	+++	+++	+++	+++
ApaL I	+++	++	+	+	Mlu I	++	+++	+++	++
Ase I	+	+++	+++	+++	Mnl I	+++	+++	+++	++
Ava I	+++	+++	+++	+++	Mse I	+++	+++	++	+
Ava II	+++	+++	++	+	Msp I	+++	+++	+++	+++
Avr II	+++	+++	+++	++	Nae I	+++	+++	+++	+
Bal I	+++	++	++	+	Nar I	+++	+++	+	+
BamH I	+	++	+++	+++	Nci I	+++	+++	++	+
Ban I	+++	+++	++	++	Nco I	+	++	+++	+++
Ban II	+++	+++	+++	+++	Nde I	+	+	++	+++
Bbv I	+++	+++	+++	+++	Nhe I	+++	+++	+++	++
Bcl I	+	+++	+++	+	Nla III	+	+	+	+
Bgl I	+	+++	+++	+++	Nla IV	+	+	+	+
Bgl II	++	+++	+++	+++	Not I	+	+++	+++	+++
Bsm I	+++	+++	+++	+++	Nru I	+	+++	+++	+++
Bsp 1286	+++	+++	++	+	Nsi I	++	++	++	++
BspH I	++	+++	+++	++	PaeR7 I	+++	+++	+++	+
BspM I	++	++	+++	+++	PfIM I	++	+++	+++	++
BspM II	++	++	+++	+++	Ple I	+++	+	+	+
BssH II	+++	+++	+++	+++	PpuM I	+++	+++	++	+
BstB I	+++	+++	++	+	Pst I	+++	+++	+++	+++
BstE II	+	++	+++	+++	Pvu I	+	++	+++	+++
BstN I	++	++	+++	+++	Pvu II	+++	+++	+++	+++
BstU I	+++	+++	++	+	Rsa I	+++	+++	+++	+++
BstX I	++	+++	+++	+++	Rsr II	+++	++	+	+
BstY I	+++	+++	++	++	Sac I	+++	+	++	+
Bsu36 I	+	++	+++	++	Sac II	+++	+	+	+
Cla I	+++	+++	+++	++	Sal I	+	+	++	+++
Dde I	++	+++	+++	+++	Sau3A I	+++	+++	+++	+++
Dpn I	+	++	+++	+++	Sau96 I	+++	+++	+++	+++
Dra I	++	+++	+	+	Sca I	+	+++	+++	++
Dra III	++	+++	+++	++	ScrF I	++	+++	+++	+++
Eae I	++	+++	++	+	SfaN I	+	+	+++	+++
Eag I	++	+++	+++	+++	Sma I	+	+	+	+
EcoN I	+++	+++	+++	+++	SnaB I	+++	+++	++	+
EcoO 109	+++	+++	+++	+++	Spe I	++	+++	+++	++
EcoR I	+	+++	+++	+++	Sph I	+	+	+++	+++
EcoR V	+	++	+	+++	Ssp I	++	+++	+++	++
Fnu4H I	+++	+++	++	+	Stu I	+++	+++	+++	+++
Fok I	+++	+++	+++	+++	Sty I	+	++	+++	+++
Fsp I	+	+++	++	++	Taq I	+++	+++	+++	++
Hae II	+++	+++	+++	++	Tth111 I	+++	+++	+++	++
Hae III	+++	+++	+++	+++	Xba I	+	+++	+++	+++
Hga I	+++	+++	+	+	Xca I	+++	+	+	++
HgiA I	+	+	++	+++	Xho I	++	+++	+++	+++
Hha I	+	+	+	++	Xma I	+++	+++	++	+
Hinc II	++	+++	+++	+++	Xmn I	+++	+++	+	+
Hind III	++	+++	+++	++					

Scoring:

+++ indicates that between 30–100% of the activity can be obtained using these conditions compared to the recommended conditions.

++ indicates that between 10–30% of the activity can be obtained using these conditions compared to the recommended conditions.

+

indicates that < 10% of the activity can be obtained using these conditions compared to the recommended conditions.

*Not recommended because of star activity

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Appendix II

Bacterial Strains, Viruses, and Plasmids

E. coli C600

E. coli C600(λ C1857)

E. coli HA3 (*purA*45, *uvrA*6, *recA*56, *srl*C300:Tn10, *gal*-6, *xyl*-7, *lacY*1 or *lacZ*4, *tsx*-1 or *tsx*-70, *tonA*2, *mal*⁻)

E. coli K-12

Salmonella typhimurium LT2

Micrococcus luteus

Bacillus subtilis

Saccharomyces cerevisiae

Bacteriophage lambda (λ)

λ DNA

pRPC245

pBR322

pUC-4K

The cultures listed above can be purchased commercially from American Type Culture Collection (ATCC), Ward's Biology, or Carolina Biological Supply Co. The plasmid pBR322 and λ DNA can be obtained from Sigma Chemical Co. The plasmid pUC-4K (or the Kan cartridge) can be obtained from Pharmacia LKB Biotechnology. The strains *E. coli* HA3, *E. coli* C600(λ C1857) and the plasmid pRPC245 are specialized constructions, and can be purchased as a set from the author for \$30.00 (to cover the cost of preparation and mailing). Please include a check or money order with each request. Address inquiries and requests to:

Holly Ahern

Department of Biological Sciences

The University at Albany

Albany, NY 12222

Appendix III

Enzymes and Buffers

Restriction Enzymes

Restriction enzymes can be purchased commercially from a number of different biotechnology companies. (See list of suppliers in appendix IV.)

Nde I

Sal I

Hind III

EcoR I

Pst I

Other Enzymes

T4 DNA Ligase

Buffers

10X KGB (potassium glutamate buffer—available from Pharmacia LKB Biotechnology)

10X Restriction enzyme reaction buffers (usually included in enzyme order)

10X Ligase reaction buffer (available with enzyme)

Appendix IV

Equipment and Supplies

Agarose Gel Electrophoresis
(mini-gel apparatus)

SDS-PAGE

Power Supply

VacuBlot Apparatus

Photography Supplies
Camera

UV Transilluminator

Autoradiography Supplies
Chemicals

Microcentrifuge

Micropipettors and Tips

Chemicals and Reagents

Nitrocellulose, other supplies
Isotope

Plasmid DNA Purification

Circle Prep Purification Kit

Chromosomal DNA Purification
The Extractor Kit

DNA Labeling and Detection

Genius Nonradioactive DNA Labeling
and Detection Kit

Bio-Rad Laboratories
Hoefer Scientific Instruments
Cabisco Biotechnology
Bio-Rad Laboratories
Hoefer Scientific Instruments
Cabisco Biotechnology
Bio-Rad Laboratories
Cabisco Biotechnology
American Bionetics (available through
Fisher Scientific)

Fotodyne (available through Cabisco
Biotechnology)
Fotodyne (available through Cabisco
Biotechnology)
Kodak Laboratory
Kodak Laboratory
Fisher Scientific
Cabisco Biotechnology
Bio-Rad Laboratories
Cabisco Biotechnology
Amresco, Inc.
Sigma Chemical Company
Cabisco Biotechnology
Boehringer Mannheim Biochemicals
Bio-Rad Laboratories
NEN Research Products

BIO 101

Molecular Biosystems, Inc.

Boehringer Mannheim Biochemicals

Suppliers

Amersham, Inc.
2636 S. Clearbrook Dr.
Arlington Heights, IL 60005

Amresco, Inc.
P.O. Box 39098
30175 Solon Industrial Parkway
Solon, OH 44139-9827

BIO 101
P.O. Box 2284
La Jolla, CA 92038-2284

Bio-Rad Laboratories
2200 Wright Avenue
Richmond, CA 94804

Boehringer Mannheim Biochemicals
9115 Hague Road
P.O. Box 50816
Indianapolis, IN 46250

Cabisco Biotechnology
2700 York Road
Burlington, NC 27215

Fisher Scientific
711 Forbes Avenue
Pittsburg, PA 15219

Hoefer Scientific Instruments
654 Minnesota Street
Box 77387
San Francisco, CA 94107

Kodak Laboratory
Laboratory and Research Products Division
Rochester, NY 14652-3512

Molecular Biosystems, Inc.
10030 Barnes Canyon Road
San Diego, CA 92121

New England Biolabs
32 Tozer Road
Beverly, MA 01915

NEN Research Products
549 Albany Street
Boston, MA 02118

Pharmacia LKB Biotechnology
800 Centennial Avenue
Piscataway, NJ 08854

Promega Biotechnology
2800 S. Fish Hatchery Road
Madison, WI 53711

Sigma Chemical Company
P.O. Box 14508
St. Louis, MO 63178-9916

Stratagene
11099 N. Torrey Pines Road
La Jolla, CA 92037

Appendix V

Additional Procedures

Southern Blot—Capillary Method

1. Electrophorese the gel and stain with ethidium bromide. Photograph the gel for a permanent record.
2. Transfer the gel to a glass baking dish. Remove unused portions of the gel with a razor blade and discard.
3. Soak the gel in 0.5 N NaOH, 1.5 M NaCl for 30 minutes at room temperature with constant agitation to denature the DNA.
4. Neutralize the gel by soaking it in 1 M Tris-Cl pH 8.0, 1.5 M NaCl for 30 minutes at room temperature with constant agitation.
5. Cut nitrocellulose or nylon membrane and five pieces of Whatman 3 MM paper to the exact size of the gel. Also cut two pieces of Whatman paper to use as “wicks.”
6. Completely wet the membrane by immersing it at a 45° angle into 10X SSC. Allow the membrane to soak for 15 minutes.
7. Wet the Whatman paper wicks with 10X SSC. Lay the wicks over an elevated glass plate in a tray, allowing the ends of the wicks to drape over the edges of the plate. (See figure A.3.)
8. Add 10X SSC to the glass tray to a level just below the elevated glass plate.
9. Place the denatured and neutralized gel on top of the wicks.
10. Center the wetted membrane on top of the gel, making sure that no air bubbles form between the gel and the membrane. If bubbles are present, smooth them out using a glass rod.
11. Center five pieces of Whatman 3MM paper on top of the membrane.
12. Place a 1–2-inch stack of paper towels, cut to the dimensions of the gel, on top of the filter paper. Place a glass plate on top. Add a weight (such as a full reagent bottle) to the top to complete the stack.
13. Allow the transfer to proceed overnight, replacing the 10X SSC in the glass tray as it is absorbed.
14. Following the transfer, remove the weight, paper towels, and filter papers from the top of the membrane.

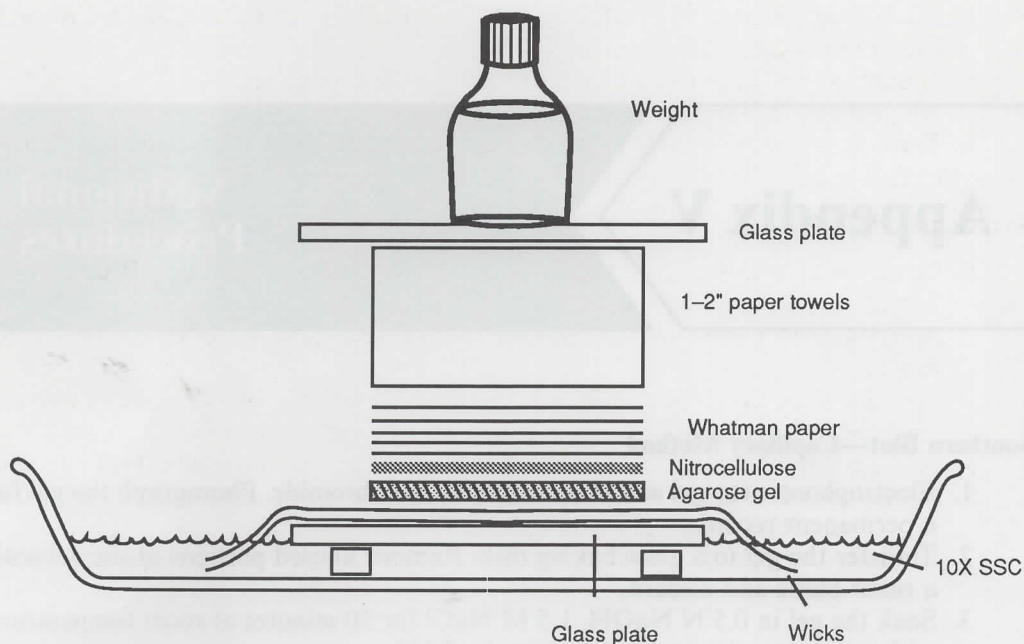


Figure A.3. Nucleic acid transfer by capillary blot.

15. Carefully lift the membrane from the gel. Wash the membrane once with 10X SSC for 20 minutes at room temperature with constant agitation. Also remove the gel and examine it under UV light to insure that all of the nucleic acids were transferred. (The gel can be restained with ethidium bromide if desired.)
16. Place the membrane with the transferred DNA face up on a piece of filter paper and allow it to air dry. Bake the DNA onto the membrane for 2–4 hours at 80°–100° C (in a vacuum oven if required).
17. Hybridize the membrane using standard techniques.

Transformation of Plasmid DNA

This method may be used in place of the colony transformation procedure.

Preparation of Competent Cells

1. Inoculate 100 ml of TY broth with 1 ml of an overnight bacterial culture. Grow the cells in a shaking waterbath at 37° C for 2–4 hours.
2. Chill the culture on ice for 10 minutes. Centrifuge at 4° C for 5 minutes at 4,000g.
3. Pour off the supernatant, and resuspend the cells in 50 ml of ice cold 50 mM CaCl_2 , 10 mM Tris-Cl pH 8.0. Incubate the suspension on ice for 15 minutes.

4. Centrifuge the suspension at 4° C for 5 minutes at 4,000g. Pour off and discard the supernatant.
5. Resuspend the cells in 7 ml of ice cold 50 mM CaCl₂, 10 mM Tris-Cl pH 8.0. Aliquot into 0.2-ml fractions.
6. The competent cells can be used immediately, stored at 4° C for up to 24 hours, or frozen at -70° C.

Transformation

1. Place the appropriate number of tubes of competent cells on ice. (If cells were frozen, allow them to thaw on ice.)
2. Add 10–100 ng of DNA directly to the competent cells. Incubate on ice for 30 minutes.
3. Transfer the tubes to a 42° C waterbath, and incubate for exactly 2 minutes.
4. Remove the tubes from the waterbath and place them on ice for 5 minutes.
5. Add 1 ml of TY broth to each tube. Incubate at 37° C for 15 minutes to 1 hour.
6. Spread 100–200 μ l of the transformed cells onto the appropriate selective media plates using the spread-plate technique.
7. Incubate the plates overnight at 37° C, then examine for the appearance of transformants.

Isolation of Prokaryotic Chromosomal DNA

1. Inoculate 100 ml of TY broth with bacteria. Incubate with shaking at 37° C until the cells reach late log phase (approximately $5-8 \times 10^8$ cells/ml).
2. Pour the culture into centrifuge tubes and spin for 5 minutes at 10,000 rpm.
3. Remove and discard the supernatant. Resuspend the pellets in 10 ml of SET buffer (20% sucrose, 50 mM Tris-Cl pH 7.6, 50 mM EDTA). Combine the two suspensions into one tube and spin for 5 minutes at 10,000 rpm.
4. Remove and discard the supernatant. Freeze the cell pellet for 15 minutes at -70° C (or 5 minutes in a dry ice-ethanol bath).
5. Thaw the pellet in a 37° C waterbath. Resuspend the cells in 2 ml of SET buffer and place the tube on ice.
6. Add 0.2 ml of lysozyme (5 mg/ml in 10 mM Tris-Cl pH 7.6, 1 mM EDTA, 10 mM NaCl) and 0.1 ml of RNase (pancreatic ribonuclease A, 10 mg/ml, in 0.1 M Na acetate, 0.3 mM EDTA, pH 4.8, preheated to 80° C for 10 minutes). Mix well the contents of the tube and incubate on ice for 15 minutes.
7. Add 0.05 ml 25% SDS and incubate the mixture at 37° C for 4 hours with constant shaking.
8. Add 0.3 ml of proteinase K (50 μ g/ml in TEN buffer) and 1.5 ml of Sevag; mix. Incubate with gentle agitation overnight.
9. Add 1 ml of distilled water and 2 volumes (10 ml) of Sevag to the tube. Invert the tube gently for 5 minutes. Centrifuge at 10,000 rpm for 5 minutes. The solution will separate into two phases: an upper aqueous phase and a lower organic phase.
10. Remove the upper phase using a wide-mouth pipette and place it into a clean tube. Repeat the extraction with Sevag two more times.

11. Following the final extraction, remove the upper phase and place it into a clean tube using a wide-mouth pipette. Add 0.2 ml of 5 M NaCl and 2 volumes of ice cold 95% ethanol. Mix the solution gently, and incubate on ice for 5 minutes.
12. Using a glass rod, spool out the precipitated DNA. Dip the rod into cold 95% ethanol to wash the DNA.
13. Place the rod into a clean tube and add TE (to dissolve the DNA). Store the purified genomic DNA at 4° C.

Isolation of *Bacillus Subtilis* Chromosomal DNA

1. Inoculate one liter of BHI broth with a culture of *Bacillus subtilis*. Incubate the cells in a 37° C shaking waterbath until the cells reach log growth (approximately 2–4 hours).
2. Centrifuge the cells for 5 minutes at 10,000 rpm. Pour off the supernatant, and resuspend the cells in a total of 20 ml TEN buffer (combining the pellets into one tube).
3. Centrifuge at 4° C for 5 minutes at 6,500 rpm. Pour off the supernatant and resuspend the cells in 10 ml of SET buffer. Add 1 ml of lysozyme and incubate at 37° C for 30 minutes.
4. Divide the cell suspension into two tubes. Add 5 ml of TEN buffer and 0.5 ml 25% SDS to each tube. Mix the tubes by inverting them gently several times.
5. Add 1 ml of 5 M NaCl to each tube, and add an equal volume of phenol. Invert the tubes gently several times for approximately 5 minutes.
6. Centrifuge the tubes at 4° C for 5 minutes at 6,500 rpm. The solution will separate into two phases, with a protein precipitate in between. Using a wide-mouth pipette, remove the upper phase without disturbing the protein and place it into a clean tube.
7. Extract the solution once with an equal volume of Sevag. Separate the phases by centrifugation. Remove the upper phase and place it into a clean tube. Place the tube on ice.
8. Add two volumes of ice cold 95% ethanol, and incubate on ice for 5 minutes.
9. Using a glass rod, spool the precipitated DNA out of solution. Rinse the DNA once in 95% ethanol.
10. Dissolve the DNA in 10 ml of TE buffer. Incubate overnight at 4° C.
11. Add 0.05 ml of RNase and incubate at 37° C for 2 hours.
12. Add 0.5 ml of proteinase K and continue the 37° C incubation for 1 hour.
13. Add 5 ml phenol and 5 ml Sevag to the solution and gently mix by inversion for 5 minutes. Centrifuge to separate the phases.
14. Remove the upper phase and transfer to a new tube. Extract the solution with an equal volume of Sevag. Centrifuge to separate the phases.
15. Remove the upper phase to a clean tube and add 2 volumes of cold 95% ethanol. Incubate the tube on ice for 5 minutes, then spool the DNA out onto a glass rod (as previously described).
16. Rinse the precipitated DNA once in 95% ethanol and then dissolve it in TE buffer. Store the DNA at 4° C.

Isolation of Yeast Chromosomal DNA

1. Inoculate 1 liter of YEPD broth (peptone 20 g, yeast extract 10 g, dextrose 20 g, per liter of distilled water) with yeast culture. Incubate at 30° C until stationary phase is reached.
2. Centrifuge the cells at 4° C for 5 minutes at 6,500 rpm. (It is easiest to harvest cells using centrifuge bottles.)
3. Resuspend the cells in a total of 60 ml of distilled water (combining the pellets). Centrifuge the cell suspension at 4° C for 5 minutes at 6,500 rpm. Decant the supernatant.
4. Freeze the cell pellet at -70° C for 15 minutes (or 5 minutes in a dry ice-ethanol bath).
5. Thaw the pellets in a 37° C waterbath. Repeat the freeze-thaw process two more times.
6. Resuspend the thawed pellets in 20 ml SET buffer, and add 1 ml of 5 M NaCl to the cell suspension. Add a 1/10 volume of 25% SDS to the solution and mix by gentle inversion. Incubate the tube at room temperature for 1 hour.
7. Incubate the cell suspension at 65° C for 1 hour. Allow the lysate to cool to room temperature.
8. Add an equal volume of Sevag to the lysate. Mix by inversion several times. Centrifuge the tube to separate the phases.
9. Using a wide-mouth pipette, remove the upper phase to a new tube. Repeat the Sevag extraction once.
10. Remove the upper phase and place it into a clean tube. Add 2 volumes of ice cold 95% ethanol to the tube, and incubate on ice for 5 minutes.
11. Using a glass rod, spool the precipitated DNA out of the solution. Rinse the DNA in 95% ethanol and then dissolve it in TE.
12. Add 0.5 ml RNase to the dissolved DNA and incubate at 37° C for 1 hour. Add 0.5 ml proteinase K and incubate for 2 hours.
13. Extract the solution once with an equal volume of Sevag. Centrifuge to separate the phases. Remove the upper phase to a clean tube.
14. Add 1/25 volume of 5 M NaCl to the solution and 2 volumes of ice cold 95% ethanol. Incubate the tube on ice for 5 minutes.
15. Spool the precipitated DNA out of solution using a glass rod. Rinse it once in 95% ethanol, and then dissolve it in TE. Store the DNA at 4° C.

Isolation of Yeast Chromosomes (1984)

1. Inoculate 1 liter of YEPD broth (yeast 20 g, yeast extract 10 g, benzimidazole 0.5 g per liter of distilled water) into yeast culture. Incubate at 30°C until stationary phase is reached.
2. Centrifuge the cells at 4°C for 5 minutes at 8,500 rpm. (It is essential to harvest cells using centrifuge bottles.)
3. Resuspend the cells in a total of 50 ml of distilled water (washing the pellet). Centrifuge the cell suspension at 4°C for 5 minutes at 8,500 rpm. Decant the supernatant.
4. Freeze the cell pellet at -70°C for 15 minutes (or 5 minutes at a dry ice ethanol bath).
5. Thaw the pellet in a 35°C water bath. Repeat the freeze-thaw process two more times.
6. Resuspend the thawed pellet in 30 ml SET buffer, and add 1 ml of 2 M NaCl to the cell suspension. Add a 1/10 volume of 25% SDS to the solution and mix by gentle inversion. Incubate the tube at room temperature for 1 hour.
7. Incubate the cell suspension at 65°C for 1 hour. Allow the lysate to cool to room temperature.
8. Add an equal volume of 20% to the lysate. Mix by inversion several times. Centrifuge the tube to separate the lysate.
9. Using a water-immersion pipette, remove 0.5 ml of the supernatant. Repeat the 20% extraction once.
10. Remove the upper phase and place it into a clean tube. Add 1 volume of ice cold 95% ethanol to the tube and incubate for 5 minutes.
11. Using a glass rod, spool the precipitated DNA out of the solution. Repeat the DNA spooling and then dissolve it in TE.
12. Add 0.1 ml of 10% SDS to the dissolved DNA and incubate at 70°C for 1 hour. Add 0.5 ml potassium acetate and incubate for 5 hours.
13. Extract the solution once with an equal volume of 20%. Centrifuge to separate the phases. Remove the upper phase to a clean tube.
14. Add 1/25 volume of 2 M NaCl to the supernatant and 1 volume of ice cold 95% ethanol. Incubate the tube on ice for 5 minutes.
15. Spool the precipitated DNA out of solution using a glass rod. Repeat it once in 95% ethanol, and then dissolve it in TE. Store the DNA at 4°C.

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